Diflubenzuron in Atlantic Cod (*Gadus morhua*)

Multiple Dose Pharmacokinetic Study

by

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Abstract

Diflubenzuron, a chitin synthesis inhibitor, is currently being used as a parasiticide for sea lice infections in farmed salmonid species. It is administered orally via medicated feed pellets. The bioavailability in Atlantic salmon, the main target species, has been estimated to 31 % of the recommended dose when administered at a water temperature of 6 °C (EMEA, 1999). Furthermore, the main route of excretion in Atlantic salmon is via bile. After administration of radiolabelled diflubenzuron, 39 % of the activity in the bile contents was found to originate from the parent compound (Horsberg and Høy, 1991). The low bioavailability combined with biliary secretion of the active drug leads to accumulation of diflubenzuron in the intestine, and substantial amounts of active compound are released into the water column adsorbed to organic particles in faecal matter. Feed spills are also a source of contamination into the environment during medication periods. Diflubenzuron is considered stable in acidic and neutral solution, and the half life in sediment has been estimated from 3-4 weeks at a water temperature of 15 °C, up to 3 months at 5 °C (IMR, 2011). Consequently, there is a risk that non-target organisms could consume diflubenzuron during and after treatment periods via organic particles from sea pens.

Little is known about the pharmacokinetics of diflubenzuron in other fish species. In this study, the standard diflubenzuron treatment (3 mg/kg once daily for 14 days) has been administered to Atlantic cod with a mean weight of 104 g (lower and upper bounds 65 and 165 g) at a water temperature of 7.7 °C, and samples of fillet and skin in natural proportions, liver, terminal colon and bile have been collected during and in the period following the medication period (day 4, 8, 12, 15, 18, 22, 29, 36 and 44). The primary objective has been to determine the tissue levels of diflubenzuron obtained in Atlantic cod, as well as the depuration half lives in the different tissues. The analytical method has been developed at NIFES for the analysis of diflubenzuron residues in Atlantic salmon fillet and skin, and uses a reversed-phase HPLC system coupled to an MS detector with negative-ion electrospray ionization. The LOD is 10 ng/g, and the LOQ is 20 ng/g for this system.

p-Chloroaniline (PCA), which is a minor metabolite of diflubenzuron in some species, has tested positive in several *in vitro* and *in vivo* carcinogenicity assays, and acts as a potent methaemoglobinemia inductor in toxic doses. A secondary objective of this study was to

evaluate whether PCA is a metabolite of diflubenzuron in Atlantic cod, and what the implications are in terms of consumer safety. The analysis of PCA content is qualitative, based on a quantitative method that was under development at NIFES at the time of this study. The fillet and skin samples from the medication period were analyzed using a reversed-phase HPLC system coupled to a tandem MS detector. This method is not accredited, but based on previous analyses of spiked samples a LOD of about 2 ng/g is expected.

During the medication period, the calculated tissue levels in fillet and skin and liver showed high variability. This is probably due to individual differences in feed consumption, and to a lesser extent differences in absorption. The median tissue levels obtained in fillet and skin and liver were 36.1 and 106 ng/g, respectively. This is very low compared to Atlantic salmon, in which a mean concentration of 2240 ng/g in fillet and skin has been found at 6 °C 1 day after standard treatment. Furthermore, no fillet and skin samples throughout the medication period exceeded the MRL value of 1000 ng/g. The tissue levels quickly dropped below the LOD in all tissues after treatment, and although there was a high uncertainty associated with the calculated depletion rates in the different tissues because of the few data points available, the calculated half lives were less than 1 day for all tissue types. PCA was not detected in any of the fillet and skin samples throughout the medication period; this, however, does not rule out the possibility that PCA could be a metabolite of diflubenzuron in Atlantic cod, because the obtained tissue concentrations of diflubenzuron were so low that the fraction of PCA that may be formed probably would be below the detection limit of 2 ng/g.

In terms of consumer safety, there is little risk associated with the consumption of wild caught Atlantic cod that may have fed on spills of medicated feed from sea pens during diflubenzuron treatment, because diflubenzuron seems to have a low gastrointestinal uptake in Atlantic cod, and the toxic metabolite PCA was not detected in fillet and skin samples.

Abbreviations

DIF	Diflubenzuron
DoF	Directorate of Fisheries, Norway
EMEA	European Medicines Agency
ESI	Electrospray Ionization
FAO	Food and Agriculture Organization of the United Nations
FKD	Ministry of Fisheries and Coastal Affairs, Norway
HPLC	High-Performance Liquid Chromatography
IMR	Institute of Marine Research, Norway
IPCS	International Programme on Chemical Safety
IS	Internal Standard
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
LOD	Limit of Detection
LOQ	Limit of Quantification
MRL	Maximum Residue Limit
MS	Mass Spectrometry
m/z	Mass-to-Charge Ratio
NA	Norwegian Accreditation
NIFES	National Institute of Nutrition and Seafood Research, Norway
NIPH	Norwegian Institute of Public Health
NOAEL	No Observable Adverse Effects Level
NTP	National Toxicology Program, US
PCA	*para*-Chloroaniline
SD	Standard Deviation
SPE	Solid-Phase Extraction
TEF	Teflubenzuron
WHO	World Health Organization

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Chapter 1 Introduction

Parasitic sea lice constitute the most severe disease problem in Norwegian aquaculture today, causing an annual economic loss which has been estimated to 131 million €, based on production statistics from 2006 (Costello, 2009). Several species within the Salmonidae family are farmed in Norway, of which Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) are produced in the highest quantities. At the end of 2011, a biomass of approximately 676 and 79 million kg, respectively, of these two species was held in Norwegian aquaculture (DoF, 2012).

The pathogens commonly found are the salmon louse (*Lepeophtheirus salmonis*) and various *Caligus* species. As the salmon louse is the cause of the most serious infections in Atlantic salmon farms in the Northern Hemisphere (Pike and Wadsworth, 1999), the following background information will focus on the salmon louse. The salmon louse is a crustacean of the copepod group. It is a host specific ectoparasite, being dependent on salmonids to complete its life cycle, and occurs naturally in sea water on the Northern Hemisphere (Boxaspen, 2006). Its life cycle consists of 10 stages, as illustrated in Figure 1.1, with moulting of the cuticula between each stage. The first two stages, nauplius I and II, are free swimming. The third, infective stage is the copepodid, which attaches itself to the salmonid and feeds on the skin, mucus and blood of the host. The chalimus stages remain attached to the host, while the preadult and adult stages are mobile on the host. Each adult female can release 200-500 eggs every 10 days during the summer, the number and interval depending on sea temperature (Heuch *et al.*, 2000). The fertilized eggs are hatched from egg strings which are attached to the host.

The damage inflicted on the host depends on the number and stage of the parasitic lice, with the mobile stages causing more severe damage (Wagner *et al.*, 2008), as well as the size and condition of the host (Finstad *et al.*, 2000). The tissue damage predisposes the fish to secondary infection, as well as disturbing the osmotic regulation. Additionally, increased cortisol levels due to stress response in the fish may lead to immunosuppression and further increase the susceptibility to secondary infection (Pickering and Pottinger, 1989). Atlantic salmon post-smolt do not seem to survive infection with more than 10 mobile lice (Holst *et al.*, 2003).

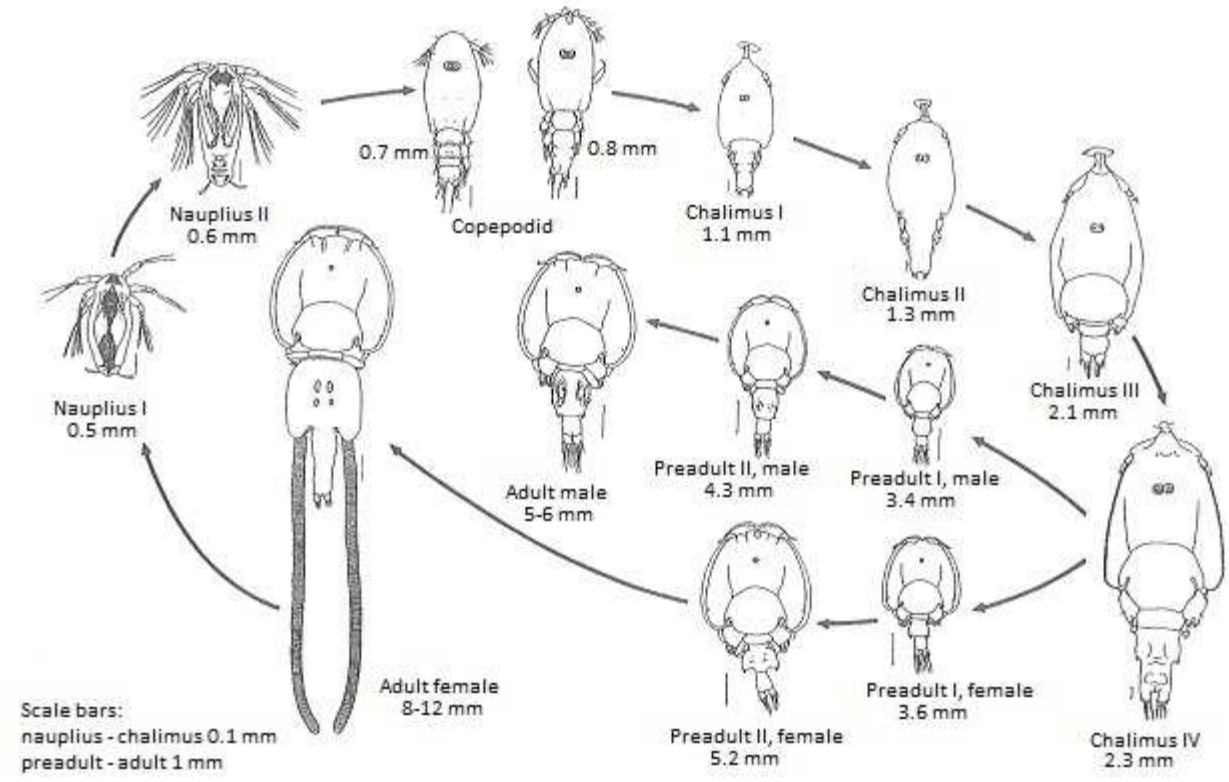

Figure 1.1 Life cycle of the salmon louse (*Lepeophtheirus salmonis*). Adapted from Schram (1993)

Salmon louse parasitism not only represents a major challenge in terms of fish welfare and economy in salmonid aquaculture, it may also present a threat to the wild salmonid population, as both wild and farmed salmonids are potential hosts to the salmon louse. The free stages of the salmon louse can spread passively over a distance up to 100 km (Asplin *et al.*, 2004), spreading the infestation bilaterally between farming facilities and the wild population of salmonids. The infectious pressure is the product of the number of fish in the system, and the number of lice per fish (Heuch and Mo, 2001). An increased biomass in salmonid farming might thus directly increase the infectious pressure to the wild population, if the number of lice is not effectively kept at a minimum in the sea pens.

In order to reduce the number of adult lice, the wrasses (*Labridae*) are being used as cleaner fish in sea pens. When the number of lice per individual reaches a threshold, chemical agents are used. The different chemical treatments available are bath treatments with organo-phosphorus compounds, pyrethroids or hydrogen peroxide, and in-feed treatment with avermectins or benzoylureas (Veterinærkatalogen, 2012, Grave *et al.*, 2004). Strict regulations are in place regarding the use of both chemical and biological measures. The goal is to keep louse levels at a minimum while at the same time minimizing the development of resistance towards any agent. An overview of the use of each chemical agent over the last few years is given in Table 1.1. The utilized amounts of different drugs are however not directly comparable, as potency differences between different drugs are not taken into account.

Table 1.1 Use of chemical agents against sea lice in Norway, listed as kg of active substance (NIPH, 2011b)

		2005	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**
Benzoylurea compounds	Diflubenzuron	-	-	-	-	1413	1839	704
	Teflubenzuron	-	-	-	-	2028	1080	26
Organo-phosphorus compounds	Azamethiphos				66	1884	3346	2437
Pyrethroids	Cypermethrin	45	49	30	32	88	107	48
	Deltamethrin	16	23	29	39	62	61	54
Avermectins	Emamectin	39	60	73	81	41	22	105
Chemical disinfectant	Hydrogen peroxide (tonnes)					308	3071	3144

As can be seen in Table 1.1, the number of chemical agents and the amount of each agent used rose steeply in 2009 and 2010 in particular, with the exception of emamectin, which was used to a lesser extent in these years. These trends were most likely due to increasing reports of reduced sensitivity to emamectin and the pyrethroids deltamethrin and cypermethrin (NIPH, 2011a), as well as new regulations lowering the threshold number of lice per fish requiring mandatory delousing treatment of aquaculture facilities (FKD, 2009). Note that emamectin and the pyrethroids are more potent compared to the other chemical agents, this means that a relatively small reduction or increase in the use of emamectin or pyrethroids will have a large impact on the consumption of other agents (Veterinærkatalogen, 2012). While

there has been some reduction in 2011 compared to the two previous years, the use is still very high compared to 2005-2008 levels. The reduction in 2011 is probably due to improved resistance control, but could also partly be explained by natural fluctuations in sea lice number and infectious pressure (NIPH, 2012) as well as an increase in the use of emamectin compared to 2010.

Due to the increasing development of resistance towards other chemical agents, the benzoylurea insecticides diflubenzuron and teflubenzuron (Figure 1.2) have recently come back into use. Their exact mechanism of action is not fully understood, but they inhibit chitin synthesis *in vivo* (Matsumura, 2010), thus disrupting the normal shedding of the cuticula and hindering the ecdysis process between growth stages. Benzoylureas act as parasiticides when they are administered over a period which includes ecdysis. They are thus ineffective against the adult stages of the salmon louse, but exhibit up to 90 % mortality in the earlier stages (Horsberg, 2000).

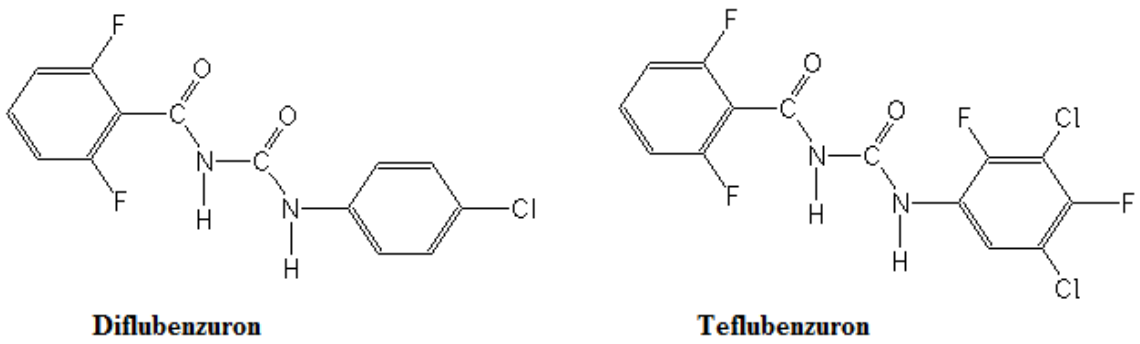

Figure 1.2 The molecular structures of the benzoylureas diflubenzuron and teflubenzuron

Diflubenzuron [1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)-urea] currently does not have a general marketing authorization in Norway, but it is available through application to the Norwegian Medicines Agency as Releeze® medicated feed pellets manufactured by Ewos. It is administered orally in a standard dose of 3 mg/kg for 14 days. It is poorly absorbed from the gastrointestinal tract of Atlantic salmon, with a bioavailability of approximately 31 % of

the recommended dose when administered at a water temperature of 6 °C (EMEA, 1999). In the same study the mean peak plasma level was reached after 24 hours (EMEA, 1999).

In order to achieve successful treatment of salmon louse infection, a minimum concentration of 900 ng/g in fillet and skin tissue is assumed to be required by the industry (pers.comm., Hege Hovland at Ewos). Tissue levels obtained in a study of Atlantic salmon following standard treatment at 6 and 15 °C can be seen in Table 1.2.

Table 1.2 Tissue levels of diflubenzuron in Atlantic salmon (600 to 1346 g) following standard treatment (EMEA, 1999)

Days after treatment	**Mean diflubenzuron residues found (ng/g)**			
	Fillet and skin in natural proportions		**Liver**	
	+ 6 °C	**+ 15 °C**	**+ 6 °C**	**+ 15 °C**
1	2240	1550	3190	2170
7	400	200	730	260
14	100	40	120	40
21	40	30	30	20

Due to the low bioavailability of diflubenzuron, the concentration in faeces will be higher compared to the original in-feed concentration, as nutrients are more readily absorbed from the gut lumen. In a study of ^{14}C-diflubenzuron in Atlantic salmon by Horsberg and Høy (1991), diflubenzuron was found to be excreted mainly via the biliary route in Atlantic salmon, and 6 hours after administration 39 % of the radioactivity in bile was the unmetabolized parent compound. Thus enterohepatic circulation also contributes to a high concentration of diflubenzuron in the faeces of Atlantic salmon.

As can be seen in Table 1.1, a considerable amount of diflubenzuron was used in 2009 and 2010. However, a substantial recent reduction is seen, and during the second half of 2011 diflubenzuron was hardly applied. During treatment, a considerable amount of active substance is released into the environment. A simplified estimate is that 31 % of the dose is absorbed from the gut, and 39 % of this is excreted unmetabolized in faeces. Based on these data, and assuming that all of the medicated feed is consumed by the target salmonids within the sea pens, approximately 81 % of the administered dose will still be released into the water column as the active substance. Diflubenzuron is only sparingly soluble in water (Table 1.3),

and the concentration in the surrounding water will be low. The majority of the active substance will enter the environment adsorbed to organic particles, either from spills of medicated feed pellets or in faeces.

These organic particles can spread over a large area, and potentially be consumed by non-target organisms. The half life of diflubenzuron in marine sediment has been estimated to be 3-4 weeks at 15 °C, and up to 3 months at 5 °C (IMR, 2011). Half life estimations of diflubenzuron in the water column and sediments are very variable, depending on the experimental design. However, for the purpose of this study it is sufficient to know that the diflubenzuron content of organic particles released from sea pens during and after delousing treatment remains stable for a relatively long time.

Table 1.3 Physico-chemical properties of diflubenzuron (JMPR, 2002) and teflubenzuron (JMPR, 1996).

	Diflubenzuron	**Teflubenzuron**
Mw	310.7 g/mol	381.1 g/mol
Water solubility (20 °C)	0.2 mg/l	0.02 mg/l
Acetone solubility	6.98 g/l	10 g/l
Hexane solubility	63 mg/l	50 mg/l
Dichloromethane solubility	1.8 g/l	1.8 g/l
Log P_{ow}	3.83	4.56

Diflubenzuron is considered relatively non-toxic in humans, with an acceptable daily intake (ADI) limit of 0.02 mg/kg body weight (JMPR, 2001). In high doses, diflubenzuron has been shown to cause haematotoxicity in various species, with dose-related formation of methaemoglobin and sulfhaemoglobin (JMPR, 2001). The oral LD_{50} levels in mice and rats are >4500 mg/kg body weight (FAO/WHO, 1996), and the no observable adverse effects level (NOAEL) for haematotoxicity after long-term exposure in mice, rats and dogs has been found to be 2.4, 2.0 and 2.0 mg/kg body weight, respectively (FAO/WHO, 1996). No evidence has been found for carcinogenicity, mutagenicity or teratogenicity for either diflubenzuron or its main metabolites (FAO/WHO, 1996). Diflubenzuron has been classified by the WHO as a substance “unlikely to present an acute hazard in normal use” (WHO, 2004), but in 2009 the classification was changed to “slightly hazardous” (WHO, 2009).

Nevertheless, diflubenzuron is approved by the WHO for use in drinking water to reduce the growth of disease spreading vectors such as mosquito larvae (WHO, 2008). After treatment with diflubenzuron, salmonids must be withheld from slaughter for a minimum of 105 degree-days (°CD) (Veterinærkatalogen, 2012). The maximum residue limit (MRL) is 1000 µg/kg, or 1000 ng/g (JMPR, 2001, Veterinærkatalogen, 2012).

Figure 1.3 illustrates the major metabolic pathways of diflubenzuron in animals. There is some concern regarding a possible mutagenic effect of *p*-chloroaniline (PCA), which is a minor metabolite of diflubenzuron. Note that only a small fraction of the *p*-chlorophenylurea is transformed to PCA (IPCS, 1996), and that the formation of this metabolite is species dependent. PCA is known to be a metabolite of diflubenzuron in pigs and rats. After oral administration of radiolabelled diflubenzuron to a pig, the PCA concentration recovered in urine represented about 0.06 % of the absorbed diflubenzuron dose (Opdycke *et al.*, 1982). In a study of diflubenzuron metabolism in rats, at most 0.01 % of the absorbed diflubenzuron dose was converted to PCA, estimated by the concentration in urine (IPCS, 1996). PCA has not been detected in Atlantic salmon treated with diflubenzuron. It is not known whether PCA is formed as a metabolite of diflubenzuron in Atlantic cod.

PCA is carcinogenic in rats and mice, and has also tested positive in several *in vitro* mutagenicity assays (IPCS, 1996). If this metabolite is present in wild population Atlantic cod which has fed on medicated feed spills, it would be necessary to evaluate the implications for consumer safety.

A PCA mutagenicity study in mice and rats has been performed by the US NTP (1989). At a dose of 200 and 400 mg/kg per day, all rats and mice died within 6 days. In groups administered 25, 50 and 100 mg/kg daily for 16 days, enlargement of the spleen was observed in rats. In mice, liver and spleen lesions were also observed at these dose levels. In a 13 week study of groups of rats administered up to 80 mg/kg, and mice administered up to 120 mg/kg, there were no deaths related to the PCA administration. Spleen enlargement and dose related secondary anemia due to methaemoglobinemia was observed. In a 103 week study, groups of mice and rats were administered a dose of 3 and 2 mg/kg, respectively, for 5 days a week. Fibrosis of the spleen was observed at this dose level, but survival did not appear to be negatively affected compared to control groups. Administration of 18 and 30 mg/kg to rats and mice, respectively, for 103 weeks increased the tumor incidence in both species.

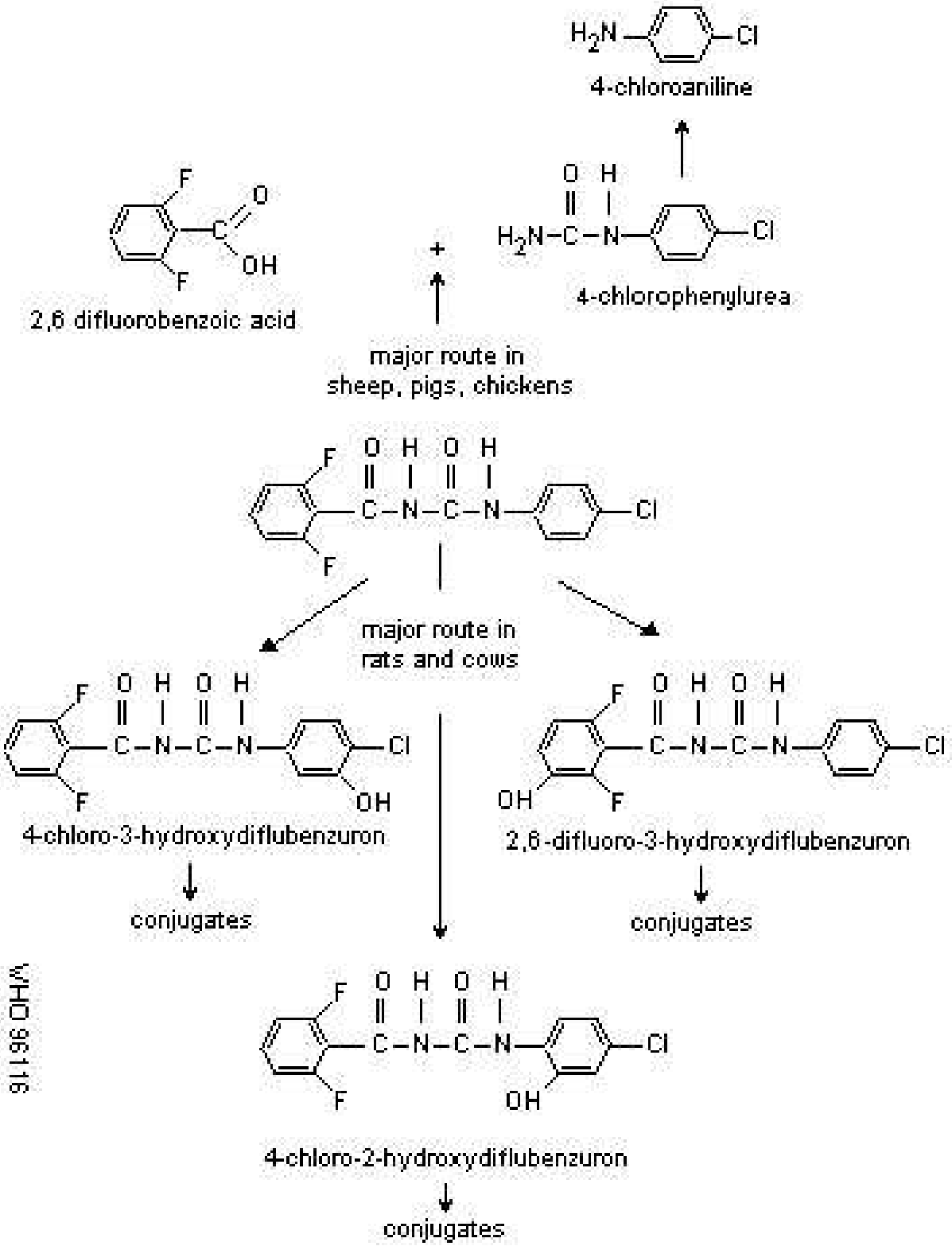

Figure 1.3 Animal metabolism of diflubenzuron (FAO/WHO, 1982)

PCA also displays acute toxicity in high doses, as it is a potent methaemoglobin inductor (IPCS, 1996). It is used in large volumes as a dye intermediate (NTP, 1989), and exposure to toxic levels has lead to life-threatening methemoglobinemia in humans, as demonstrated by several case studies from dermal and inhalation exposure to occupational workers (Pizon *et al.*, 2009).

For practical reasons, systemic treatment of large quantities of fish is usually administered via feed when this is possible. However, this means that it is not possible to control the amount of drug consumed by individuals. The individual dose will depend on appetite as well as the overall condition of the fish. Sick individuals have reduced appetite, making it less likely that they will receive a sufficient dose to benefit from the treatment. However, with today's low infection threshold levels for treatment with chemical agents (FKD, 2009) as well as improved preventive husbandry practices, sea louse infections heavy enough to significantly reduce the appetite of the fish are unlikely to occur.

After consumption of medicated feed, the extent of absorption of diflubenzuron, which has a moderate to low bioavailability, will depend on gastric emptying and intestinal passage time. These factors are affected by the formulation of the feed, as well as the physiology of the species and the individual. Other physiological factors are also important, such as the gastrointestinal surface area available for absorption, and the activity of any active uptake mechanism.

Differences in drug consumption as well as absorption contribute to a wide inter-individual variability in drug levels after oral administration to fish, and consequently the range of residue levels normally found between individuals given the same treatment is wider for fish compared to mammals (NicGabhainn *et al.*, 1996). This necessitates a higher number of samples, with ten or more samples at each interval generally regarded as a minimum (Treves-Brown, 2000). For Atlantic cod, which is a territorial species, aggressive individuals may dominate during feeding and consume a relatively large amount of the available feed. Additionally, Atlantic cod display irregular eating habits, and may eat a large portion on one day and perhaps not any food on the following day (pers.comm., Hari Rudra at IMR). These factors may further contribute to wide interindividual variability.

There is currently considerable interest in the development of Atlantic cod farming. The stock of Atlantic cod in Norwegian farming facilities in recent years is shown in Table 1.4. If the

biomass of farmed Atlantic cod should increase in the future, the increase in host individuals could potentially lead to an increase in the infective pressure of parasitic copepods on Atlantic cod, similarly to the effect that has been proposed for Atlantic salmon (Heuch and Mo, 2001).

Table 1.4 Stock of Atlantic cod (1000 individuals) in Norwegian aquaculture at the end of each year (DoF, 2011).

	2002	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**
Cod, hatched	1512	5546	8642	12176	15382	16652	24685	17893	11461
Cod, wild caught	301	240	219	177	72	27	206	3	1
Total	1813	5786	8861	12353	15454	16679	24891	17896	11462

Atlantic cod is known to be a host to several copepod species, exemplified by the cod worm (*Lernaeocera branchialis*) which infects the gills and is considered to be a potential problem in cod-farming (Khan *et al.*, 1990), *Clavella adunca*, which infects skin, fins and gills, and several sea lice of the *Caligus* family (*C. curtus*, *C. elongatus*) which infect the skin and have been reported cause disease problems in farmed Atlantic cod in Norway (Johnson *et al.*, 2004).

If parasitic copepod infections in Atlantic cod should escalate in parallel with a possible future increase in the farming of this species, it would be necessary to evaluate the efficacy of the available parasiticides in Atlantic cod.

Aim of this study

The primary aim of this study is to determine the uptake, distribution and elimination rates of diflubenzuron in Atlantic cod based on data from multiple dose oral treatment with diflubenzuron, equivalent to the regime used to treat infection with sea lice in Atlantic salmon.

Additionally, the study aims to qualitatively determine any presence of *p*-chloroaniline in the fillet and skin samples, in order to determine whether diflubenzuron is metabolized to *p*-chloroaniline to any extent in Atlantic cod.

The results will be used to evaluate the potential toxicity for humans through consumption of wild Atlantic cod which has fed on medicated feed spills from salmonid farming.

Diflubenzuron will also be evaluated as a potential antiparasitic agent for use in Atlantic cod.

Chapter 2 Materials and methods

2.1 Chemicals

The medicated feed used was Releeze® from Ewos. The feed consists of pellets which contain diflubenzuron at a final concentration of 0.6 g/kg. The benzocaine preparation used to kill the fish was Benzoak® from ACD Pharmaceuticals, 200 mg/mL.

Acetonitrile HPLC grade, heptane HPLC grade, and acetone HPLC grade were all from Sigma-Aldrich. Diethyl ether analytical grade, tetrahydrofuran HPLC grade, 25 % aqueous ammonium solution HPLC grade and formic acid 98-100 % purity HPLC grade were all from Merck. Dichloromethane HPLC grade was from Riedel-de Haën. 18.0 MΩ purified water was used for all analytical purposes.

Diflubenzuron (CAS number 35367-38-5) and teflubenzuron (CAS number 83121-18-0) for calibration curve and internal standard were both analytical grade from Aldrich.

2.2 Preparation of standard solutions

Three separate stock solutions were prepared, namely diflubenzuron for standard curve, diflubenzuron for control samples and teflubenzuron for internal standard, by dissolving 10.00 ± 0.04 mg in 10 mL tetrahydrofuran, to a concentration of 1.0 mg/mL. These solutions were stored in amber glass vials at fridge temperature (4 °C), with a durability of one year.

Working solutions of 10 µg/mL diflubenzuron for standard curve, diflubenzuron for control samples and teflubenzuron for internal standard were prepared by dilution of the stock solutions in two steps with acetonitrile:distilled water (1:1). First 100 µL of the stock solutions were diluted to 10 mL, and then 500 µL of the diluted solutions were further diluted to 10 mL. The working solutions were durable for three days at fridge temperature (4 °C).

2.3 Experimental conditions

2.3.1 Fish and holding conditions

Triploid Atlantic cod (*Gadus morhua*) was used, with a mean length of 22.4 ±1.2 cm and a mean weight of 104 ± 20 g (upper and lower bounds 165 and 65 g), delivered from the Parisvatnet field station of the Institute of Marine Research. The water temperature was 7.7 °C (± 0.2 °C) throughout the period.

2.3.2 Feeding

A controlled experimental study was conducted, administering medicated feed pellets to Atlantic cod for a period of 14 days. The feed pellets contained 0.6 g of diflubenzuron per kg, and the administered dose was 0.5 % of the total weight of the fish per day based on the average weight of the fish at the beginning of the treatment period. This gives a total dose of 3 mg diflubenzuron per kg fish per day. The feed was administered *ad libitum*, which means that the actual dose varies according to the feeding behaviour of the individual fish. All of the medicated feed was however consumed each day. After the medication period was completed at day 14, the fish were not fed for a few days before normal feeding was resumed.

2.3.3 Sample collection

Samples of 10 fish from the medicated group and 6 fish from the control group were collected during and after the treatment period, as indicated in Table 2.1.

Table 2.1 Intervals of sampling in relation to the medication period, counted in days from commencement and cessation of the medicated period, and numbering of samples collected from the medicated group on each day.

Sample collection	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Medication, start	4	8	12	15	18	22	29	36	44
Medication, end				1	4	8	15	22	30
Samples	110-119	120-129	130-139	140-149	150-159	160-169	170-179	180-189	190-199

The fish from the medicated group and the control group were collected from separate tanks into separate containers, and killed with a lethal dose of benzocaine solution added to the containers. Samples of fillet and skin in natural proportions, liver, and terminal colon were taken from both groups. Additionally, bile samples were collected when available. The samples from the medicated group were all collected and analyzed individually, with the exception of the bile samples which were accumulated into one or two group samples for each sample day, depending on the amount of sample material available. The control group samples for each tissue type were collected in one group sample for each sample day. All sample material was stored at -20 °C between sample collection and further analysis.

2.3.4 Sample preparation

The crude samples were homogenized using a Polytron PT 2100. To prevent cross-contamination between samples, the apparatus was cleaned with soap, water and acetone between samples. The samples were also ground up in decreasing order of concentration, starting with the control group samples and subsequently counting backwards from sample collection 9 (day 44/30). Due to loss of sample material in the apparatus, the Polytron could not be used for small samples such as the individual liver and colon samples. These samples were instead roughly homogenized using a scalpel with a disposable blade.

2.4 Analytical method

The method for extraction and detection of diflubenzuron that is utilized in this study has been developed by NIFES for analysis of diflubenzuron residues in samples of fillet and skin in natural proportion from salmonids, using teflubenzuron as internal standard (IS), and is accredited by NA for this purpose (NIFES, 2004). The method has not previously been validated for samples of different matrix types or from different species, but experience has shown that the method is robust against variations in matrices.

To extract diflubenzuron and IS from the sample matrices, acetone was used, in which diflubenzuron and teflubenzuron are both highly soluble (Table 1.3). As the solubility of both compounds is higher in more polar organic solvents, a double extraction with heptane was performed in order to remove fat-soluble contaminants from the acetone solution without removing the analytes. The samples were further purified by automated solid-phase extraction (SPE) before separation of diflubenzuron and teflubenzuron by reversed-phase high-performance liquid chromatography (HPLC) combined with mass spectrometry (MS) detection and quantification.

A method for qualitative and quantitative determination of *p*-chloroaniline content is currently under development at NIFES, but at the time of this study it has not yet been available for quantitative determination of *p*-chloroaniline content. It has however been possible to reanalyze the samples of fillet and skin from the medication period on the HPLC/MS/MS instrument intended for this method, and although any *p*-chloroaniline response cannot be quantified, it is possible to qualitatively determine whether this metabolite is formed to any extent in Atlantic cod.

2.5 Sample extraction

From the sample material, 1 g wet weight (± 0.04 g) was measured out for extraction. Where the sample mass was less than 1 g, the result was corrected to g^{-1} after quantification. The samples were put in 25 ml plastic centrifuge tubes and internal standard was added, 50 ng/g teflubenzuron (100 µl 0.5 µg/ml teflubenzuron in acetonitrile:water (1:1)). After allowing 10 minutes for the internal standard to be absorbed into the sample matrices, 5 ml of acetone was added to the tubes. The samples were stirred for approximately 1 minute on a whirl mixer,

and sonicated for 10 minutes. The tubes were then centrifuged at 3500 rpm (relative centrifugal force of 2465 *g*) for 3 minutes, and the supernatant was transferred to 10 ml glass centrifuge tubes. Fat was extracted from the solution by adding 1 ml of heptane, stirring the tubes for approximately 30 seconds on a whirl mixer, and centrifuging the tubes at 2500 rpm (relative centrifugal force of 1258 *g*) for 2 minutes. The upper heptane layer was discarded, and the process was repeated. The acetone solution was then evaporated to dryness under a gentle stream of nitrogen gas at 40 °C.

The method was originally developed for salmon fillet and skin matrix, which is more lipid rich compared to cod fillet and skin. Despite this, the cod fillet and skin samples did pass the measures of internal validity. There were however some problems in the extraction steps for both the colon and liver matrices. Some but not all colon samples separated into the acetone and heptane phase, which means that it was not possible to extract fat from all colon samples. This could be due to low fat content in some of the samples. The colon samples that did separate were extracted twice with heptane as described above. None or very few of the liver samples separated into the acetone and heptane phase, and where there was a phase separation emulsion was visible between the phases. For fear of removing the analyte in the process, heptane was not removed from any of the liver samples. The following steps of the method were conducted as described below for the liver and colon samples.

The bile samples were fully soluble in acetone, but upon addition of heptane a viscous layer formed in the bottom of the tubes, with no phase separation between acetone and heptane. The bottom layer probably consisted of insoluble bile salts. After vigorous stirring on a whirl mixer and centrifugation at 2500 rpm (1258 *g*), the soluble fraction was collected and evaporated to dryness before further purification and analysis as described below. This did not give good results, and a further attempt was made at dissolving the bottom fraction in dichloromethane, in which diflubenzuron and IS are known to be readily soluble (Table 1.3). After thorough mixing, the bile fraction was centrifuged to the bottom as described above, and the dichloromethane fraction was collected and evaporated to dryness before proceeding with the following steps.

2.6 Automated solid-phase extraction (ASPEC)

Reversed-phase solid-phase extraction is used in order to purify samples by separating different molecules in a mixture based on differences in polarity. The dissolved sample is passed through a solid silica column and eluted with a non-polar solvent. The silica column retains polar molecules, while non-polar molecules pass through the column and are discarded. By gradually increasing the polarity of the solvent, the purified analyte is finally flushed through the column and collected (Hennion, 1999).

In this study, a Gilson ASPEC XL4 system with Agilent silica columns was used. Heptane was used as the non-polar solvent, and the solvent polarity was increased by mixing heptane with a gradually increasing proportion of diethyl ether.

The dried samples were dissolved in 5 ml of heptane, and transferred to 20 ml glass tubes for purification by automated solid-phase extraction. The columns were initially eluted with pure heptane, and then heptane:diethyl ether 5:95, 10:90 and 40:60 v/v. The collected eluate was evaporated to dryness under a gentle stream of nitrogen gas at 40 °C, and dissolved in 250 µl of 75:25 acetonitrile:water solution. The samples were then filtered through a 0.45 µm syringe filter, and transferred to 2.0 ml HPLC sample vials with 250 µl glass inserts.

2.7 Calibration curve

For each analysis run of the fillet and skin, liver and colon samples, a 5-point calibration curve was prepared by spiking control samples N1 - N5 with 20, 35, 50, 60 and 75 ng/g of diflubenzuron (0.5 µg/ml diflubenzuron in acetonitrile). In order to assess method validity, two control samples were also spiked at LOD and LOQ levels, 10 and 20 ng/g, respectively, from a separate control solution of 0.5 µg/ml diflubenzuron in acetonitrile. A blank control sample and a blank sample without matrix were also analyzed.

For the bile samples, too little sample and control material was available to make the standard curve as described above. The analysis of the bile samples was thus initially considered qualitative. It was however decided to attempt quantification by making a reduced 3-point standard curve using the LOQ sample of 20 ng/g as the lowest point, with two additional points at 50 and 75 ng/g respectively. This left just enough control material for an additional blank sample. The standard curve thus obtained will not be ideal as the standard curve and

LOQ sample should be independent, but it could give a rough estimate of the diflubenzuron content of the bile samples.

2.8 High-performance liquid chromatography (HPLC) separation with mass spectrometry (MS) detection

2.8.1 High-performance liquid chromatography (HPLC)

High-performance liquid chromatography is a highly efficient method for separation of compounds. The sample is dissolved in a liquid mobile phase, and a pump provides high pressure as the mobile phase passes through a column which contains the stationary phase. The HPLC column is tightly packed with uniformly sized particles (≤ 10 μm), which allow high-resolution separation, but require a high pressure in order to drive the mobile phase through (Miller, 2005).

Different compounds are separated based on the strength of their interactions with the stationary phase, which leads to characteristic retention times. In reversed-phase HPLC, the stationary phase is non-polar and the mobile phase is moderately polar (Miller, 2005). This leads to a fast elution of polar molecules, and increasing retention times for less polar molecules.

Finally, a detector is connected to the system in order to monitor the eluate and allow direct identification and quantification of the analytes that are separated by the column.

2.8.2 Electrospray ionization mass spectrometry (ESI-MS)

Mass spectrometry is used to detect, identify and quantify the analyte after separation in the HPLC column. When the dissolved analyte is eluted from the column, it must be ionized prior to detection. Electrospray ionization is used to transform the dissolved analyte to gaseous molecular ions. A small flow of the mobile phase containing the analyte is passed through a capillary needle, and a potential difference of 3-6 kV is applied between the needle and a cylindrical electrode nearby (Williams and Fleming, 2008). The potential difference

transforms the liquid to a fine mist of highly charged droplets, the charge depending on the sign of the voltage. A drying gas is passed through the spray to remove the solvent and release the molecular ion. The molecular ion is then subjected to a magnetic field in the MS detector, which separates the ions based on their mass-to-charge (*m/z*) ratios. For the quantitative analysis of diflubenzuron, negative-ion ESI coupled with a quadrupole MS detector is used.

A quadrupole detector consists of four parallel metal rods, with a direct voltage superimposed on a radio-frequency potential between the two opposite pairs (Williams and Fleming, 2008). Ions are injected in the center, in the direction of the rods. The ions travel at a constant velocity, in a wave pattern determined by the fluctuating potential, such that under a given set of conditions ions of only one *m/z* value are transmitted to the detector (Williams and Fleming, 2008).

2.8.3 Tandem MS

Tandem MS or MS/MS is a two-stage system. In the first stage the ion of interest is isolated based on its mass-to-charge ratio, and daughter ions are formed which are then separated in an MS detector in the second stage (Miller, 2005). The mother compound can be identified based on its characteristic daughter ions. In this study, a triple quadrupole system is used. The first quadrupole transports the molecular ion into the second quadrupole, which contains an inert gas and functions as a collision cell for the second stage of ionization, without any mass selection (Miller, 2005). The daughter ions are then selected and detected in the third quadrupole.

2.8.4 Equipment for quantitative analysis of diflubenzuron

The samples were separated by reversed-phase HPLC using a Hewlett-Packard HP-1100 autosampler, quaternary pump (G1311A) and column heater with an Asahipak ODP-50 4D column measuring 4.6 x 150 mm packed with C-17 polyvinyl alcohol octadecyl 5 µm particle size packing material.

The mobile phase was 25 % 10 mM aqueous ammonium hydroxide and 75 % acetonitrile at a flow rate of 0.7 ml/min, with an expected retention time of approximately 5 and 6 minutes for diflubenzuron and teflubenzuron, respectively. The injection volume was 20 µl.

The MS detector was a Hewlett-Packard Agilent 1100 MSD quadrupole, coupled with negative-ion electrospray ionization.

The software used to control the HPLC/MS system and process the acquired data was Agilent ChemStation for LC and LC/MS systems, revision A08.03. To ensure that the chromatogram peaks are assigned correctly to diflubenzuron and teflubenzuron, qualifier ions are assigned to each peak. For diflubenzuron, the quantified ion is 379,0 m/z and the qualifier ion at 359,0 m/z should appear at the same retention time with a peak value in the range of 32-48 % of the quantified peak.

For this system, the detection limit (LOD) of diflubenzuron is 10 ng/g, and the quantification limit (LOQ) is 20 ng/g.

2.8.5 Equipment for qualitative analysis of *p*-chloroaniline

For the detection of *p*-chloroaniline, HPLC-MS/MS with electrospray ionization was used. The samples were separated by reversed-phase HPLC using an Agilent 1200 series system, with an Agilent Eclipse Plus C18 column measuring 2.1 x 100 mm packed with dimethyl-n-octadecylsilane 1.8 µm particle size packing material.

The mobile phase was 75 % acetonitrile and 25 % formic acid (1 % aqueous solution) at a flow rate of 0.20 ml/min, with an expected retention time of approximately 1.8 minutes for *p*-chloroaniline. The injection volume was 5 µl.

Positive-ion ESI was used at the retention times of *p*-chloroaniline and diflubenzuron, while negative-ion ESI was used at the retention time of teflubenzuron, as this combination has been found to produce the smoothest peaks for each of the analytes. The MS detector was an Agilent 6410 triple quadrupole. For identification of the compounds, the characteristic transitions were 128 → 111, 315 → 141, and 379 → 158 for *p*-chloroaniline, diflubenzuron and teflubenzuron, respectively. The software controlling the HPLC/MS/MS system was

Agilent MassHunter Workstation, and the data processing program was Agilent MassHunter Quantitative Analysis.

For this system, spiked samples down to a *p*-chloroaniline level of roughly 2 ng/g have been found to be clearly detectable. Therefore 2 ng/g is considered the LOD of *p*-chloroaniline in this study.

2.9 Method validation

2.9.1 Validation criteria

Criteria for acceptance of analytical results are based on those applied to the NIFES accredited method (NIFES, 2004). The retention time for diflubenzuron and teflubenzuron in samples should not be more than ±10 % different from the retention times in spiked samples. The standard curve should have a correlation coefficient of ≥ 0.95. Diflubenzuron should not be detected in the blank sample. The LOD sample should be positive for diflubenzuron, and the LOQ sample should show retrieval within ± 33 % of the expected value, or 20.0 ± 6.6 ng/g. The qualifier ion peaks are also verified visually for each data point. There are no set limits for the slope of the standard curve, but the calculated slope has been observed over time for the accredited analysis at NIFES and values in the range of 0.91-1.69 are expected for analysis of salmon fillet samples.

2.9.2 Analytical validation series

Prior to analysis of the sample material, the method and skill of the analyst was evaluated by performing the previously described method using ten blank samples of fillet from Atlantic salmon spiked with a known concentration of 20.0 ng/g diflubenzuron. The results were assessed according to the aims of validity stated in section 2.9.1.

Chapter 3 Results

3.1 General considerations

Concentrations below LOQ level cannot be regarded as accurate measurements. However, to achieve consistency, it was decided to classify measurements below 5 ng/g as negative, while measurements between 5-10 ng/g were assigned an arbitrary value of 10 ng/g. Measurements between LOD and LOQ level are reported without modification.

The standard curve linearity has only been accredited in the range of 20-75 ng/g, but experience has shown that the linear correlation continues in a higher concentration range. However, any error in the calculated slope will have a progressively higher impact as the concentration rises.

3.2 Results from analytical validation series

The results from the spiked blank validation series, as described in Section 2.9.2, are shown in Table 3.1.

Table 3.1 Results from analytical validation series

Sample	Diflubenzuron (ng/g)	Error (%)
1	16.3	-18.50
2	17.4	-13.00
3	21.5	7.50
4	19.6	-2.00
5	16.3	-18.50
6	17.1	-14.50
7	21.1	5.50
8	19.4	-3.00
9	18.7	-6.50
10	19	-5.00
Mean	18.64	-6.80
SD	1.85	
SD (%)	9.9	

The mean retrieved concentration was 18.64 ng/g, which is equivalent to -6.80 % of the expected value. The standard deviation was 1.85 ng/g or 9.9 %. The individual results are all within the expected range of error of the method at LOQ level, which is ±33 % or ±6.6 ng/g.

3.3 Validation criteria for each series

While the validation criteria, as described in Section 2.9.1., are considered to be met by all analytical series from the fillet and skin matrix, there are some validation problems with results from both the liver and colon matrices. Due to the small sample mass available, it was not possible to run a reanalysis of the two series (5 and 8) which did not meet the validation criteria.

In the first attempt to analyze liver samples, all samples were weighed in at 1 g wet weight. This resulted in very high noise levels in the MS spectrum. In an attempt to resolve this issue, the sample size was reduced to 0.5 g. This gave a cleaner result in the MS spectrum, and all liver samples were subsequently weighed in at 0.5 g. Some of the colon samples were less than 1 g. The calculated concentration for all samples weighing less than 1 g was corrected to ng/g after analysis.

As can be seen in Table 3.2, the standard curve calculated for series 5 (liver samples) has a slope *m* of 1.805, which is quite high compared to the expected range of 0.91-1.69 for salmon fillet samples as described in Section 2.9.1, and a correlation coefficient *R* of 0.915 which is well below the limit of 0.95 for the accredited method. However, as the calculated LOQ concentration falls within the expected range, the results from this series are included in the present study. Series 6 (liver samples) yielded LOD measurements at LOQ level, which is twice the expected value. However, as the other validation criteria are met, this series is also included.

Series 8 (colon samples) did not give a clear standard curve, as the estimated slope *m* of 34.071 reflects. The LOD and LOQ samples are also far out of range. This means that the estimated concentrations in this series cannot be assumed to be accurate, and the results from this series will not be included in the present study. Series 9 (colon samples) has a slope of 1.767, which is higher than expected. However, as the other calculated values are within range and the correlation coefficient is high, this series is included. Series 10 (colon samples)

has one LOD parallel of 22.4 ng/g, but as all other criteria are met the results of this series are also included.

Validity parameters for the analysis of bile samples are not presented here, as the analysis failed to yield any usable results due to poor and inconsistent retrieval of internal standard and no detection of diflubenzuron in any samples. The results obtained are however presented in Section 3.6. Experimental data for the other matrices are given in Appendix 1, and standard curves for each analytical series are given in Appendix 2.

Table 3.2 Validation of analytical series: values marked in red are not in accordance with the validation criteria, and are discussed in the text

Matrix	Series	Samples	m	R	LODa	LODb	LOQa	LOQb
Fillet and skin	1	110-159	1.154	0.997	6.6	8.2	23.2	22.5
	2	160-179	1.241	0.999	10.0	11.3	21.1	21.9
	3	180-189	1.102	0.997	10.3	11.4	20.1	20.8
	4	190-199	1.239	0.991	9.7	10.4	19.2	19.4
Liver	5	110-139, 163-169	1.805	0.915	13.7	16.0	18.6	20.2
	6	140-162	0.997	0.999	21.1	21.4	24.2	19.7
	7	170-199	1.405	0.998	9.8	5.4	17.7	17.3
Colon	8*	110-149	34.071	0.926	43.7	50.1	51.7	62.2
	9	150-159, 165-169, 175-179, 185-189, 195-199	1.767	0.998	13.0	17.1	19.7	25.3
	10	160-164, 170-174, 180-184, 190-194	1.180	0.999	11.9	22.4	19	19.3

* Series not included in the present study

3.4 Diflubenzuron concentration in fillet and skin, liver and colon samples

As a normal distribution cannot be assumed for the population, the data are presented using median points and a 25-75 percentile distribution. This is a robust model which reduces the impact of any potential outliers. The raw data, median points and interquartile range for fillet and skin and liver tissue at all sample dates are depicted graphically in Figure 3.1 and 3.2. The analysis of the colon samples from day 4, 8, 12, and 15 was rejected because the standard curve and calculated LOD and LOQ concentrations did not meet the validation criteria, but the raw data, median points and interquartile range found on the rest of the sample days are shown in Figure 3.3. Additionally, the diflubenzuron MS response for each sample day is shown in Figure 3.4, but this figure does not give any precise quantitative data because the MS response has not been quantified by a standard curve or corrected according to the internal standard response.

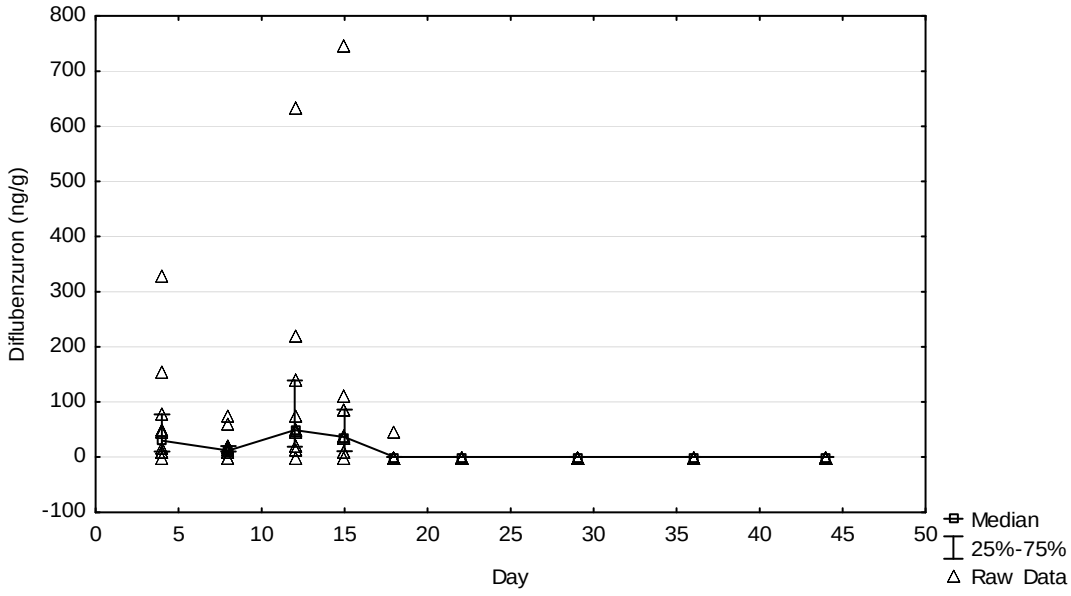

Figure 3.1 Diflubenzuron in fillet and skin samples

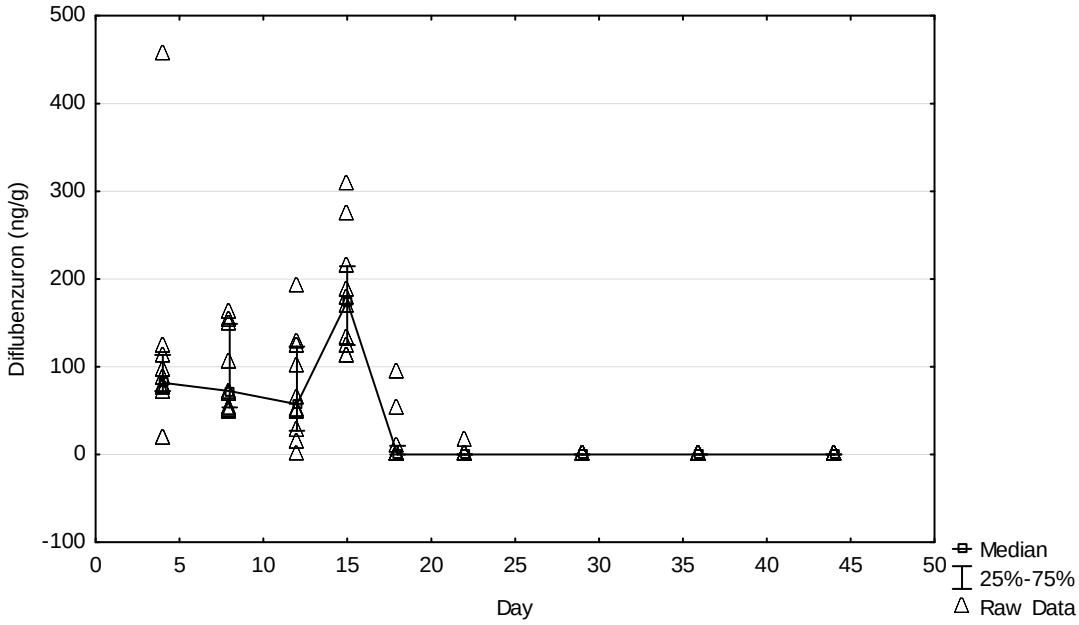

Figure 3.2 Diflubenzuron in liver samples

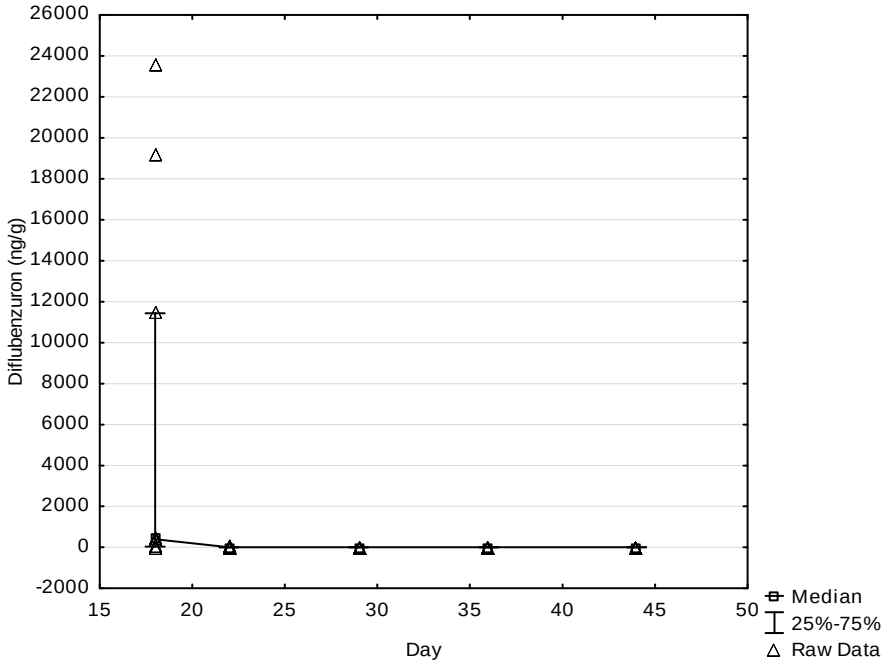

Figure 3.3 Diflubenzuron in colon samples

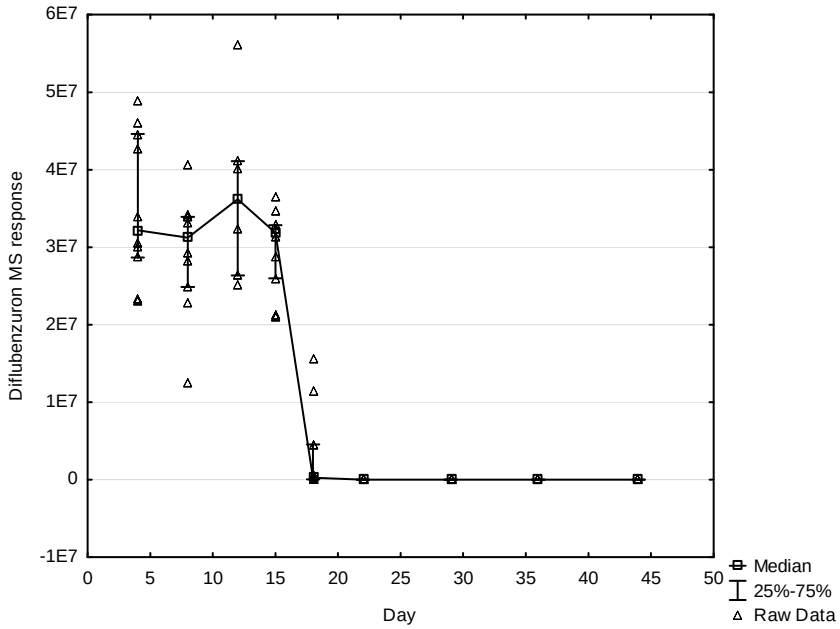

Figure 3.4 Diflubenzuron MS response in colon samples throughout the study

3.5 Depletion rates of diflubenzuron

In order to calculate the depletion half-life of diflubenzuron in various tissues, a steady state model followed by one-phase exponential decay is used. In a study of oral administration of diflubenzuron to Atlantic salmon at 6 °C, the mean peak plasma level was obtained after approximately 24 hours (EMEA, 1999). Assuming that this is comparable to the pharmacokinetics in Atlantic cod, as the bioavailability is low in both species, the steady state should persist up to day 15, which is 24 hours after the last administered dose. In order to test whether the samples taken on day 4, 8, 12 and 15 are significantly different, a Kruskal-Wallis one-way analysis of variance was performed. This is a non-parametric test of variance, and the obtained *p* value gives the probability that the compared data sets originate from a population with the same median value (Kruskal and Wallis, 1952).

In order to prove that the sample populations are different with a 95 % confidence level, the *p* value must be ≤ 0.05.

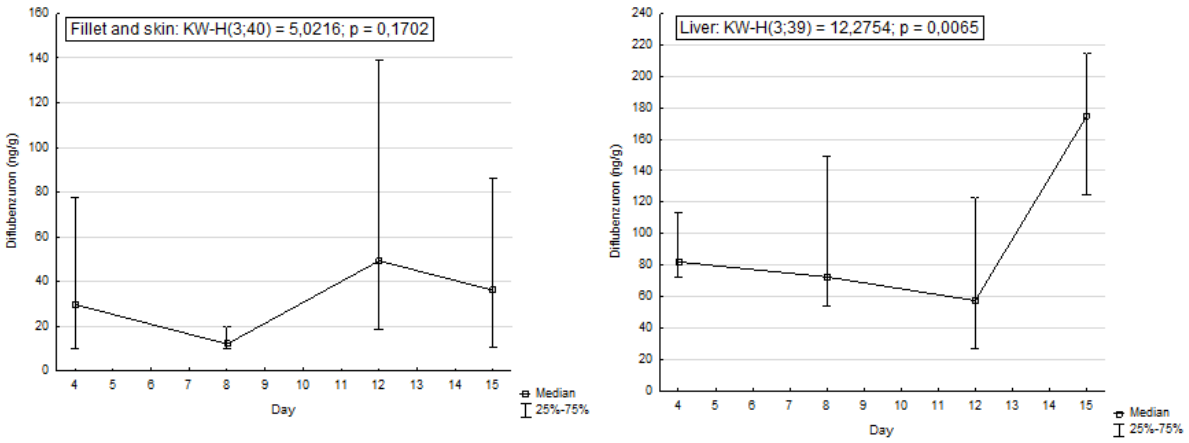

Figure 3.5 Kruskal-Wallis analysis of variance for fillet and skin and liver samples

As can be seen in Figure 3.5, the *p* value for the fillet and skin samples is 0.1702. This is not sufficiently low to reject the steady state model, and the half-life will be calculated based on a steady state through day 4 to 15.

For the liver samples in Figure 3.5, however, the *p* value is 0.0065, which means that the sample populations are too different to assume a steady state model. This appears to be due to the higher levels measured on day 15. Rather than basing the initial concentration of drug on a steady state throughout the medication period, the day 15 measurements are taken as the initial concentration, as the highest levels were found on this date.

As the day 18 measurements are the only data for initial concentration available, the depletion half-life in colon is based on day 18 as the initial steady state concentration.

Note that the half-life calculation is based on the mean concentration, which is why the graphs in Figures 3.7-3.9 do not follow the median points that are marked on the sample days. The mean is easily affected by the presence of outliers. Because of this, it was necessary to evaluate any potential outliers in the data sets used to calculate the initial steady state concentrations.

There are three extreme values in the colon samples on day 18 (23 542, 19 163 and 11 429 ng/g), as shown in Figure 3.3. Even though previous measurements are not available, these are included in the calculation of the steady state concentration based on the higher MS response seen in previous samples during the medication period (Figure 3.4).

The liver samples on day 15 are quite evenly distributed, but with two measurements some distance from the 75 percentile limit, as can be seen in Figure 3.2. However, when these values are included, the mean concentration is approximately equal to the median concentration, as can be seen in the graph in Figure 3.8. Therefore these values were not considered as outliers, and all samples from day 15 were included in the steady state calculation.

As the steady state for the fillet and skin samples is assumed to persist throughout days 4-15, these data were pooled and a median and interquartile range calculated for the pooled data set, as shown in Figure 3.6. The calculated median value is 36.1 ng/g, and the mean concentration is 82.6 ng/g when all data points are included. The outlier interval is defined with a minimum of

75 percentile limit + (outlier coefficient x interquartile range)

and a maximum of

75 percentile limit + (2 x outlier coefficient x interquartile range)

Because the population is skewed, there is no need to determine a lower outlier interval. An outlier coefficient of 3 is applied. Looking at the raw data in Figure 3.6, there is one outlier at 329.1 ng/g (day 4), as well as two extreme values at 633.3 ng/g (day 12) and 744.8 ng/g (day 15), which exceed the defined outlier interval.

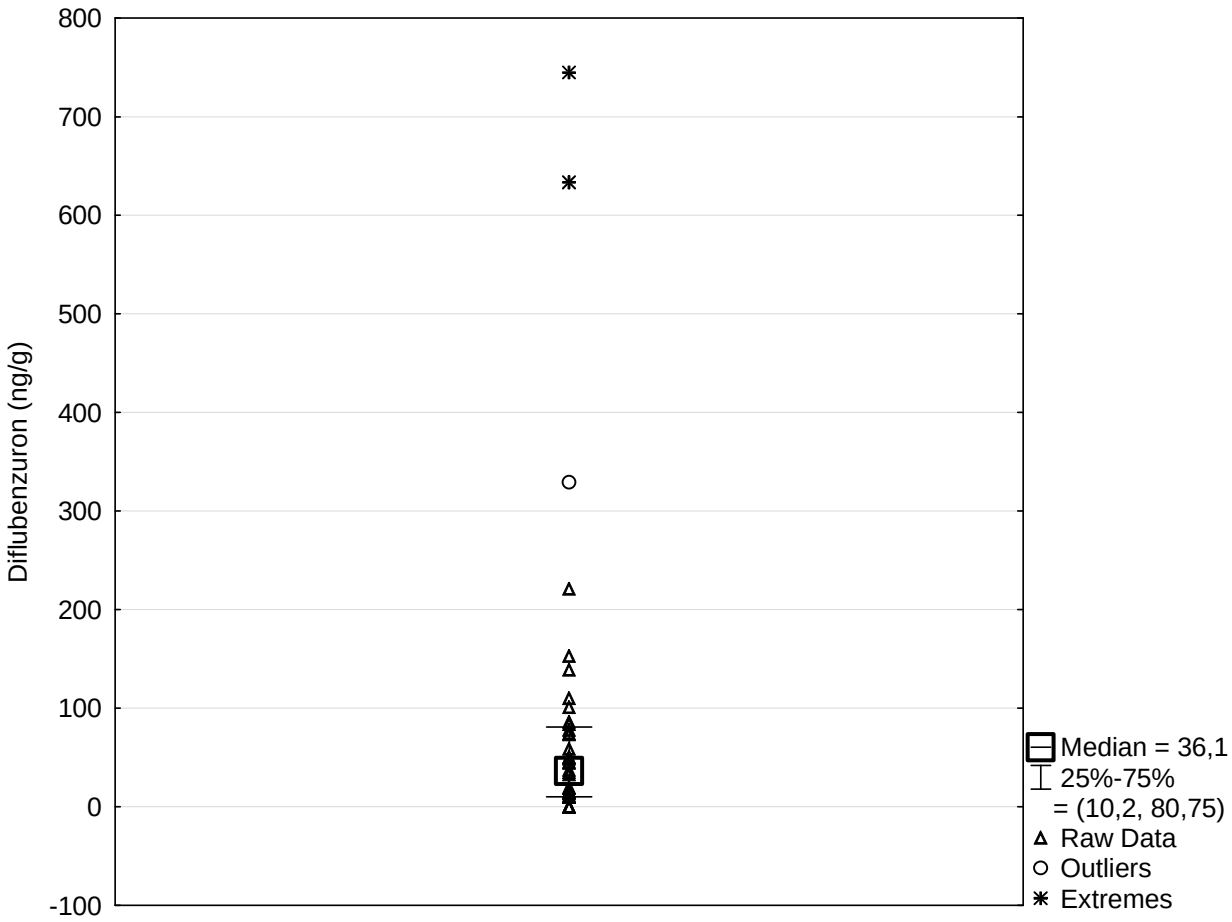

Figure 3.6 Distribution of fillet and skin samples on day 4-15

Removing the outlier and extreme values in Figure 3.6 from the data set used to calculate the depletion half-life gave a steady state concentration of 43.8 ng/g, which is closer to the median value of 36.1 ng/g, and improved the goodness of fit (R^2).

By removing outliers in the high concentration range during the steady state, the mean steady state concentration is reduced, leading to an increase in the estimated half-life.

Even though the median concentration is zero on all sample days from day 18 and onwards for fillet and skin and liver samples, and from day 22 and onwards for colon samples, none of the positive samples in the data sets after the initial steady state are treated as potential outliers. This is because the individual positive values are relatively low and have a small impact on the mean concentration, as the majority of the samples are negative. Furthermore, using the mean concentration with the single positive values included was found to give a

narrower 95 % confidence interval and improve the goodness of fit (R^2) for the calculated half-life compared to a model using the median concentration of zero.

The data used to calculate the depletion half-life in each tissue are given in Table 3.3.

Table 3.3 Data for calculation of steady state concentration and depletion half life

Fillet and skin samples – steady state										
Day	Diflubenzuron concentration (ng/g)									
4	0	329.1*	15.2	77.8	153.0	44.2	50.3	10.0	10.0	10.8
8	0	10.0	0	19.8	10.0	15.6	58.8	73.4	10.0	14.1
12	221.1	45.3	13.1	0	633.3*	18.6	48.6	49.9	74.3	138.8
15	86.0	35.0	37.2	10.0	10.4	110.2	744.8*	83.7	33.0	0
Fillet and skin samples – depletion rate										
Day	Diflubenzuron concentration (ng/g)									
18	0	0	0	0	0	0	0	46.5	0	0
22	0	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0	0
36	0	0	0	0	0	0	0	0	0	0
44	0	0	0	0	0	0	0	0	0	0
Liver samples – steady state										
Day	Diflubenzuron concentration (ng/g)									
15	111.4	186.8	111.4	132.0	178.6	274.6	308.4	170.6	214.6	124.6
Liver samples – depletion rate										
Day	Diflubenzuron concentration (ng/g)									
18	10.0	52.8	0	0	0	0	0	0	94.0	0
22	0	0	0	0	0	0	0	0	0	16.8
29	0	0	0	0	0	0	0	0	0	0
36	0	0	0	0	0	0	0	0	0	0
44	0	0	0	0	0	0	0	0	0	0
Colon samples – steady state										
Day	Diflubenzuron concentration (ng/g)									
18	23542	407.6	35.1	493.7	12.2	11429	0	19163	390.6	78.6
Colon samples – depletion rate										
Day	Diflubenzuron concentration (ng/g)									
22	0	20.7	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0	0
36	0	0	0	0	0	0	0	0	0	0
44	0	0	0	0	0	0	0	0	0	0

* Omitted values

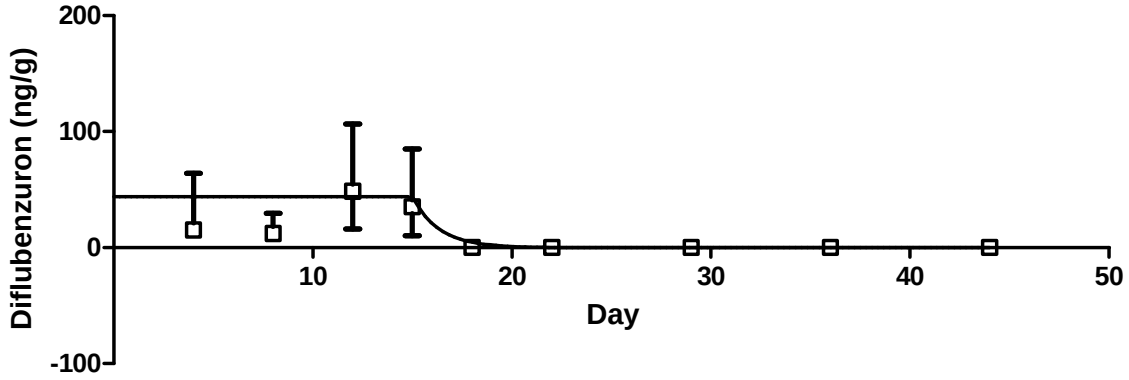

Figure 3.7 Depletion half-life of diflubenzuron in fillet and skin

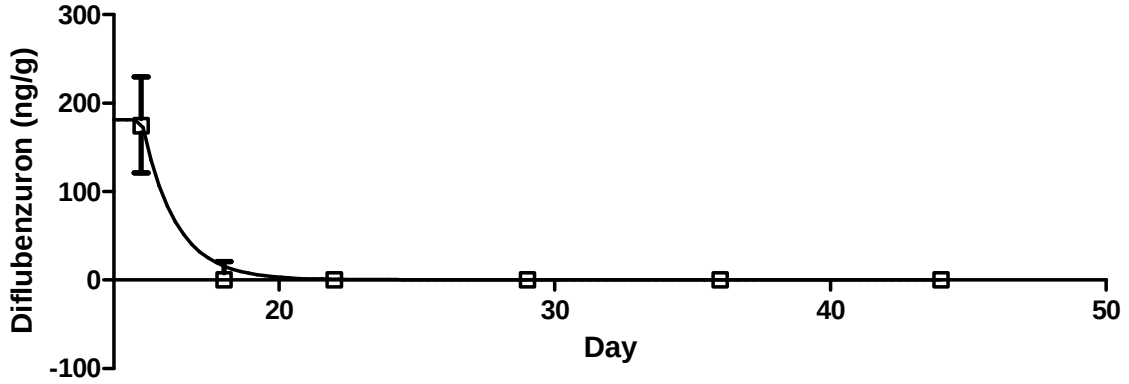

Figure 3.8 Depletion half-life of diflubenzuron in liver

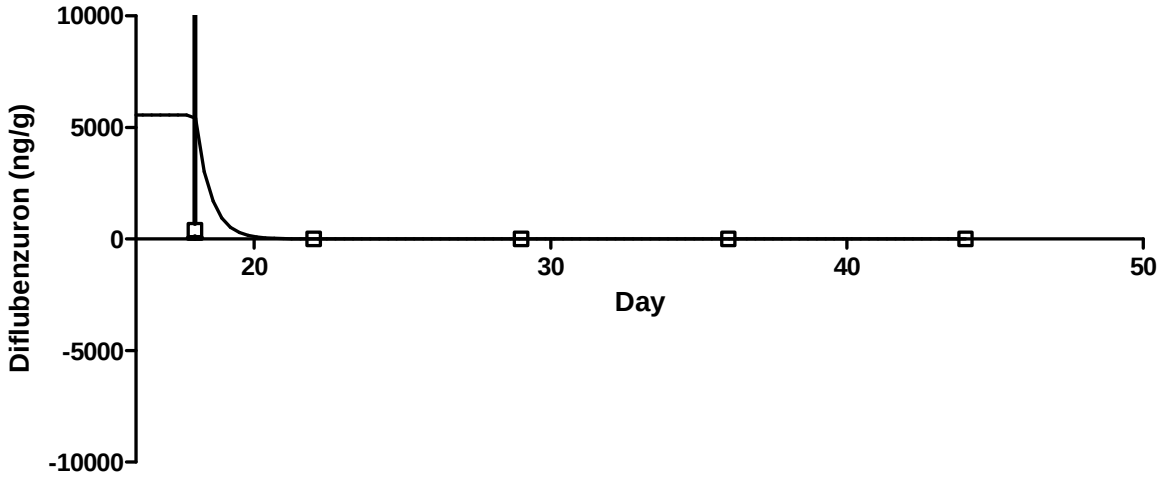

Figure 3.9 Depletion half-life of diflubenzuron in colon

Table 3.4 Depletion rates in different tissues

	Half-life (days)	R^2	95 % confidence	All negative at day
Fillet and skin	0.9	0.79	0.21 - +∞	22
Liver	0.8	0.84	0.54 - 1.91	29
Colon	0.4	0.25	0.00 - +∞	29

The calculated depletion half-lives are given in Table 3.4. They are quite similar in fillet and skin and liver, in the range of 0.8-0.9 days. In the colon, the calculated depletion rate is faster, but there is also a higher uncertainty associated with this rate as illustrated by the lower R^2 value and wider 95 % confidence interval. The calculated depletion rates are fast compared to the known pharmacokinetics in Atlantic salmon, which takes more than 21 days for the mean concentration to reach LOQ levels (Table 1.2). There is however a high degree of uncertainty associated with all the depletion rates calculated, as illustrated by the 95 % confidence intervals listed in Table 3.4. This is probably because the initial steady state concentrations were low, and the tissue concentrations quickly dropped below the detection limit. The high variability in the calculated tissue concentrations on each sample day may also contribute to the uncertainty of the depletion rate estimate.

Table 3.4 shows that fillet and skin tissue is the first to have all negative samples, on day 22, which is 8 days after the last administered dose, and liver and colon samples are all negative on day 29, or 15 days after the last administered dose. However, the liver sample size was reduced to 0.5 g due to high noise in the MS spectrum, and the calculated concentration subsequently corrected to ng/g. Because of this, diflubenzuron concentrations close to LOQ level in liver samples could go undetected, and the depletion half-life estimation in liver tissue could be too short.

3.6 Analysis of bile samples

As described in Section 2.5, upon addition of heptane to the acetone solution the bile samples formed a poorly soluble layer in the bottom of the tubes. Analysis of the soluble fraction was completed, and showed retrieval of internal standard from 5 of the 12 samples from the medicated group, but all samples were negative for diflubenzuron. There was retrieval of both diflubenzuron and internal standard in the spiked samples for the 3-point standard curve.

A second attempt was made at extraction of diflubenzuron from the bile fraction with dichloromethane. This time the spiked samples were negative for diflubenzuron, but the teflubenzuron peaks were still present. The same 5 samples from the medicated group were positive for internal standard. Diflubenzuron was not detected in any samples. Results from extraction with both solvents are shown in Table 3.5.

Table 3.5 Data from analysis of bile samples

	Heptane				Dichloromethane			
Sample	**Area DIF**	**M DIF (ng/g)**	**Area IS**	**M IS (ng/g)**	**Area DIF**	**M DIF (ng/g)**	**Area IS**	**M IS (ng/g)**
Blank	0	0	0	0	0	0	0	0
Blank matrix	0	0	16103	50.0	0	0	50032	50.0
LOQ a	8229	17.5	21886	50.0	0	0	52827	50.0
LOQ b	7950	17.7	20952	50.0	0	0	58627	50.0
N1 (50 ng)	18405	41.7	20591	50.0	1968	0	48756	50.0
N2 (75 ng)	26415	79.2	15564	50.0	3529	0	53518	50.0
1: 110-119	0	0	0	50.0	0	0	0	50.0
2: 130-139	0	0	0	50.0	0	0	0	50.0
3: 140-149	0	0	0	50.0	0	0	0	50.0
4: 150-154	0	0	25692	50.0	0	0	49552	50.0
5: 155-159	0	0	16015	50.0	0	0	51231	50.0
6: 160-169	0	0	16153	50.0	0	0	58351	50.0
7: 170-174	0	0	9026	50.0	0	0	34380	50.0
8: 175-179	0	0	0	50.0	0	0	0	50.0
9: 180-184	0	0	0	50.0	0	0	0	50.0
10: 185-189	0	0	0	50.0	0	0	0	50.0
11: 190-194	0	0	12054	50.0	0	0	56451	50.0
12: 195-199	0	0	0	50.0	0	0	0	50.0

3.7 *para*-Chloroaniline in fillet and skin samples

The HPLC sample vials from series 1 (fillet and skin samples 110-159) were kept at -20 °C after HPLC/MS analysis, and reanalyzed for *p*-chloroaniline in the HPLC/MS/MS system at a later time. All samples were negative for *p*-chloroaniline. This means that if there is any presence of *p*-chloroaniline in the fillet and skin samples, the concentration must be lower than 2 ng/g.

Chapter 4 Discussion

4.1 Validity of analysis in relation to different matrices

As can be seen in Table 3.2, the validity of the analytical series seems to depend on the matrix type that is analyzed. This could be due to differing levels of contaminants that may raise the noise level in the HPLC/MS spectrum, or due to adsorption or complex formation between components in the matrix and diflubenzuron or added standard. In order to illustrate this, the internal standard response for each sample type is plotted against the sample number in Figure 4.1. Each sample is added the same amount of internal standard (50 ng), therefore the internal standard response ideally should be independent of which matrix type is analyzed.

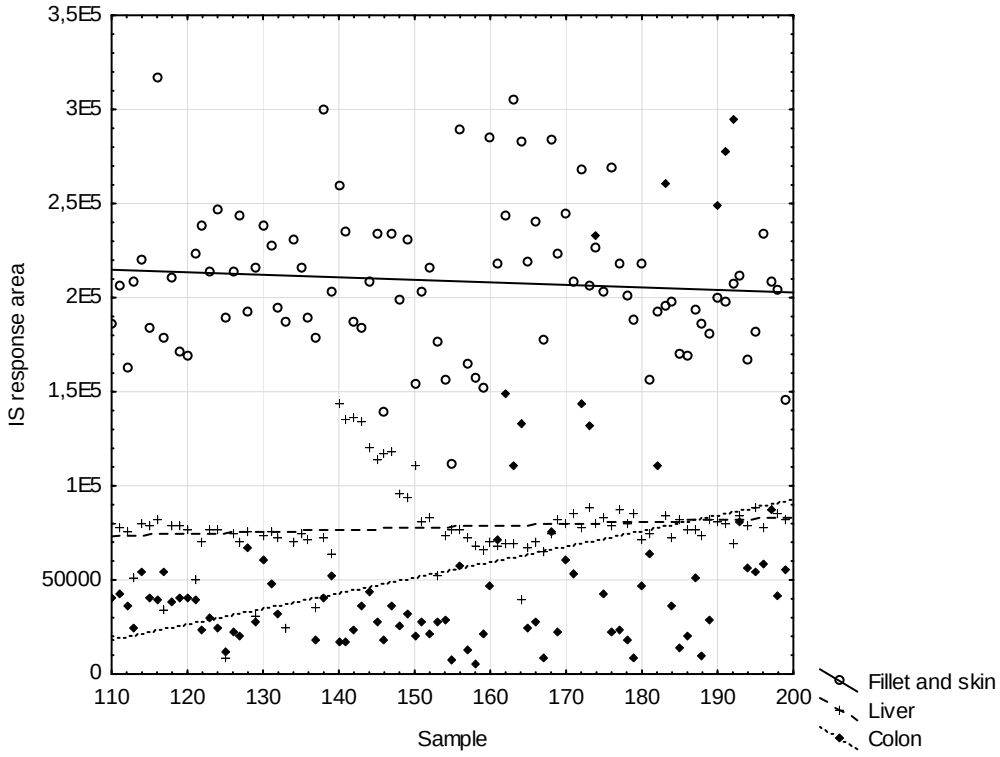

Figure 4.1 Internal standard response in different matrices

It is clear that the fillet and skin samples give a consistently much higher internal standard response compared to liver and colon samples, and colon samples have the lowest and most variable internal standard response. This corresponds to the results on the validity parameters presented in Table 3.2, in which analysis of fillet and skin samples show the highest validity, liver samples are intermediate and colon samples have the poorest validity. This must be taken into account when interpreting the results from the liver and colon samples.

In the bile samples, presented in Table 3.5, there appears to be a significant interaction between the matrix and the added diflubenzuron and internal standard. This is clear because several samples do not yield any internal standard signal upon extraction with either solvent. The internal standard signal is also relatively low. Internal standard response is depicted graphically for both solvent types in Figure 4.2.

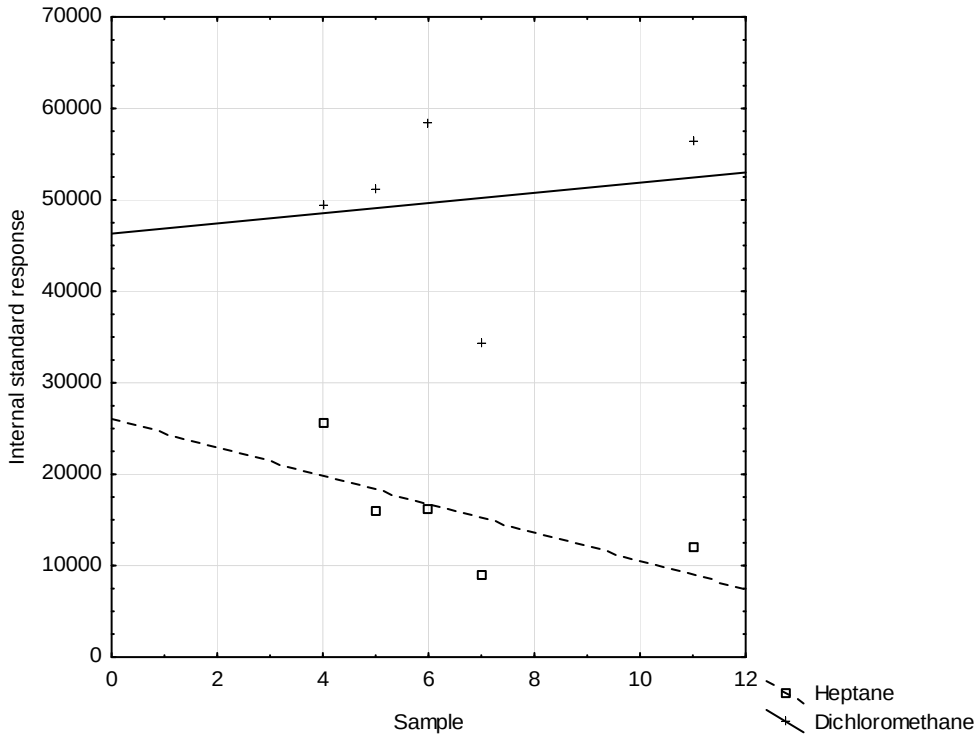

Figure 4.2 Internal standard response in bile samples

It appears that although the samples were extracted with heptane prior to dichloromethane, the dichloromethane samples still show a larger internal standard response. The diflubenzuron

response in the spiked control samples for the standard curve, as seen in Table 3.5, is however almost gone in the dichloromethane samples. This could be due to a weaker interaction between diflubenzuron and the matrix components compared to that between teflubenzuron and the matrix, leading to diflubenzuron being almost completely removed from the matrix in the first extraction with heptane, while teflubenzuron could be retained to a higher extent and subsequently available at a higher concentration in the second extraction with dichloromethane. The higher teflubenzuron response after the second extraction could also be due to a higher level of noise in the heptane MS spectrum compared to the dichloromethane spectrum, as contaminants could have been removed from the sample in the preceding heptane extraction step. The latter theory is supported by the example spectra in Appendix 3 (Figure A.23-A.28). The baseline noise levels of the spectra from the heptane extraction lie approximately in the region of 20 000-30 000, while the corresponding levels from the dichloromethane extraction lie around 2 300-2 700.

4.2 Bioavailability

In order to compare the bioavailability of diflubenzuron in Atlantic cod to that of Atlantic salmon, the main target species, Table 4.1 presents the median concentration in samples of fillet and skin and liver from day 15 and following in relation to the corresponding mean data for Atlantic salmon, as previously shown in Table 1.2.

Table 4.1 Median tissue levels obtained in Atlantic cod (65 to 165 g) compared to mean tissue levels in Atlantic salmon (600 to 1346 g) after standard treatment (EMEA, 1999)

Days after treatment	**Diflubenzuron residues found (ng/g)**					
	Fillet and skin in natural proportions			**Liver**		
	Atlantic cod	**Atlantic salmon**		**Atlantic cod**	**Atlantic salmon**	
	+ 7.7 °C	**+ 6 °C**	**+ 15 °C**	**+ 7.7 °C**	**+ 6 °C**	**+ 15 °C**
1	34	2240	1550	106	3190	2170
7	0	400	200	0	730	260
14	0	100	40	0	120	40
21	0	40	30	0	30	20

The median levels in Atlantic cod fillet and skin and liver are 1.5 and 3.3 %, respectively, of the corresponding mean levels in Atlantic salmon at the closest water temperature (6 °C). There is however a large size difference between the two groups of fish, which may contribute to the differing tissue levels. Another study with Atlantic salmon in a smaller weight class (391 to 870 g) given standard treatment by gavage at 15 °C found somewhat lower concentrations in samples of fillet and skin, namely 389, 99.6 and 21.4 ng/g at 1, 4 and 7 days post treatment, respectively (EMEA, 1999). Table 4.2 compares these data to the fillet and skin concentration obtained in Atlantic cod in the present study.

Table 4.2 Median concentration of diflubenzuron in Atlantic cod (65 to 165 g) fillet and skin samples compared to corresponding tissue levels in Atlantic salmon (391 to 870 g) after standard treatment (EMEA, 1999)

Days after treatment	**Diflubenzuron residues found (ng/g)**	
	Atlantic cod	**Atlantic salmon**
	+ 7.7 °C	**+ 15 °C**
1	34	389
4	0	99.6
7	0	21.4

At day 1 post treatment, the diflubenzuron concentration in Atlantic cod is 8.7 % of the concentration in Atlantic salmon. The data in Table 4.2 are not directly comparable to the present study, as the Atlantic salmon were held at a higher temperature (15 °C) and would probably obtain higher tissue levels at 6 °C. Furthermore, unlabeled diflubenzuron was administered to the Atlantic salmon up until the last medication day, when the radilabeled compound was administered (EMEA, 1999). This means that the actual tissue concentration of diflubenzuron may have been higher, because only the radiolabeled compound was detected through radio-HPLC. In view of this, the apparent difference in bioavailability between Atlantic salmon and Atlantic cod is too great to be accounted for by size differences alone.

The bioavailability of diflubenzuron has been found to vary depending on the administered dose in several species. In Atlantic salmon, the bioavailability ranges from only 3.7 % 12 hours after oral administration of 75 mg/kg by gavage at a water temperature of 8 °C

(Horsberg and Høy, 1991), up to 31 % which is the calculated bioavailability of the recommended dose of 3 mg/kg at 6 °C (EMEA, 1999). Based on these values, the uptake mechanism in Atlantic salmon is considered dose dependent and saturable (EMEA, 1999). The low tissue levels obtained in Atlantic cod in this study compared to Atlantic salmon reference levels could thus be due to species dependent differences in the capacity of the uptake mechanism, with a lower saturation concentration of the uptake mechanism in Atlantic cod. An alternative explanation is a higher metabolism capacity in Atlantic cod compared to Atlantic salmon. A higher first pass metabolism could reduce the bioavailability of the dose, leading to lower steady state levels, and higher metabolism and/or excretion rates could lead to quick depuration of diflubenzuron from Atlantic cod during and after treatment.

Looking at Figure 3.1, it is clear that while the median concentration of diflubenzuron in fillet and skin samples is very low (36.1 ng/g) during the treatment period, there are four samples in the range of 200-750 ng/g. Knowing that Atlantic cod is a territorial species, where dominating individuals may compete for more than their share of the administered feed, and that the feed intake of Atlantic cod may vary greatly from day to day (pers.comm., Hari Rudra at IMR), it is possible that the high extreme values may be more representative for the actual uptake of diflubenzuron in Atlantic cod than the low median concentration. All of the administered medicated feed was consumed quickly each day, and it is possible that there was competition for feed between individuals during the medication period.

The calculated median steady state concentration in fillet and skin samples throughout the treatment period is 36.1 ng/g. This is only 4 % of the minimum tissue concentration that is assumed to be required for efficacy against salmon lice, namely 900 ng/g (pers. comm., Hege Hovland at Ewos). If the bioavailability of diflubenzuron in Atlantic cod is controlled by a dose dependent saturable mechanism, it is highly unlikely that an increase in dose can mediate the low tissue levels, and diflubenzuron will probably be ineffective as a treatment for parasitic copepods in Atlantic cod. Furthermore, it is environmentally undesirable to increase the dose of diflubenzuron, as most of the administered dose will reach the environment as the unaltered parent compound. From an environmental perspective, an alternative agent with a higher bioavailability in Atlantic cod is likely to be a better choice.

4.3 Tissue distribution

As can be seen in Figure 4.3, there is a consistently higher diflubenzuron concentration in liver tissue compared to fillet and skin. These measurements are however grouped together, and do not show the distribution in the individual fish. To illustrate the distribution within individual fish, the fraction of diflubenzuron in each tissue was calculated by dividing the measured concentration in fillet and skin with the total concentration in both tissues in each sampled fish. This relationship is illustrated in Figure 4.4, showing the median fraction of diflubenzuron in fillet and skin tissue to be about 20-25 % of the total. This means that, in individual samples, the median liver concentration is 4-5 times higher than the median fillet and skin concentration.

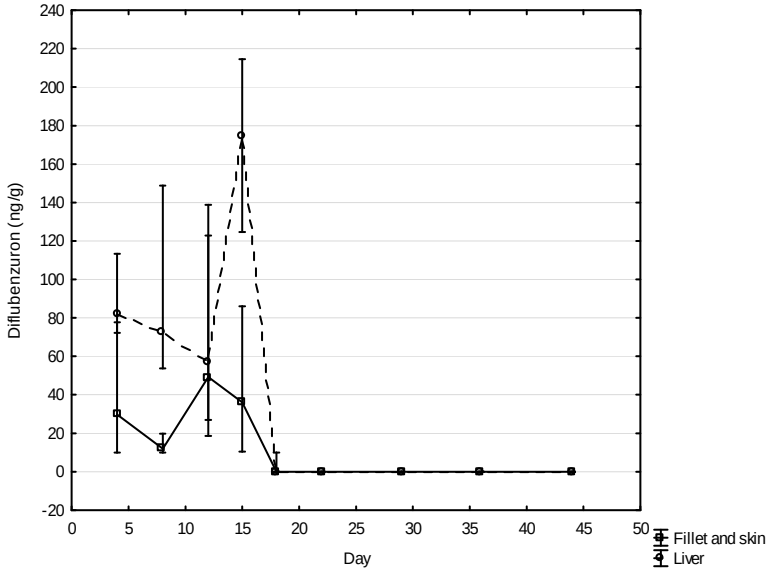

Figure 4.3 Diflubenzuron concentration in liver and fillet and skin tissues

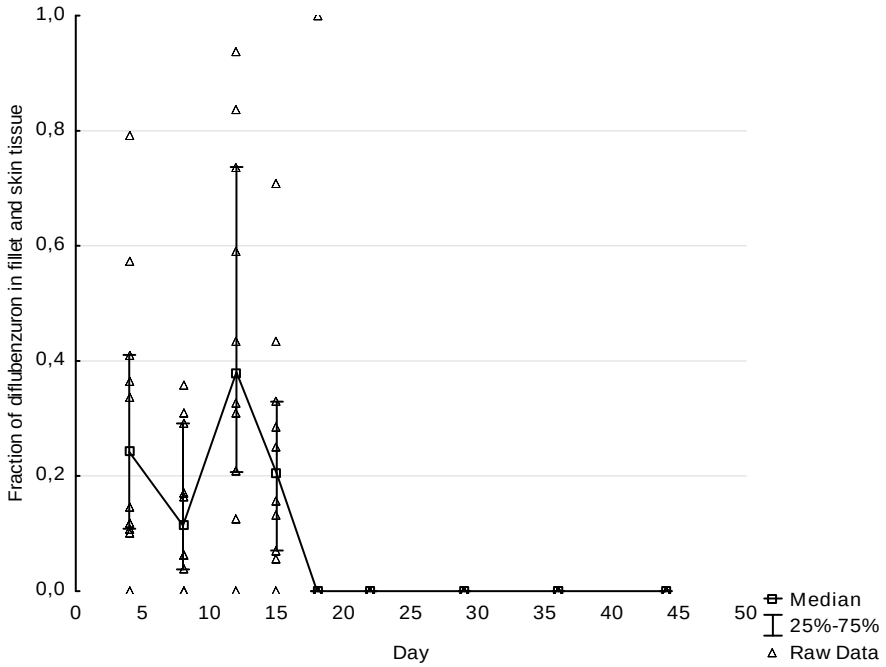

Figure 4.4 Distribution of diflubenzuron between liver and fillet and skin tissues

Atlantic cod fillet is lean compared to Atlantic salmon fillet. This is because Atlantic cod have their main fat stores in the liver and not in the fillet, as is the case for Atlantic salmon. Horsberg and Høy (1991) however found a higher concentration in the liver of Atlantic salmon compared to fillet at all sample dates, which is comparable to the distribution found in Atlantic cod, as shown in Figure 4.4.

From Figure 4.3, it is clear that there is a high variability in both the fillet and skin and the liver samples during the treatment period, even though a steady state should theoretically occur throughout sample days 4-15, such that all samples taken within this period should originate from a group with the same median value. The variance tests in Section 3.5 (Figure 3.5) show that there is a 17 % chance that the muscle samples in this period originate from a steady state population with the same median. However, in fish a wide range of residue levels between individuals given the same treatment is not uncommon (NicGabhainn *et al.*, 1996). This is particularly true for orally administered drugs. The main reason is variation in feed intake, but interindividual variation in absorption may also contribute, particularly for drugs with low bioavailability such as diflubenzuron (Treves-Brown, 2000).

For the liver samples however, the probability that samples from day 4-15 originate from a population with the same median value is only 0.65 %. The low *p*-value appears to be due to the high concentrations measured on day 15. There is no overlap between the 25-75 percentiles on day 15 compared to both day 4 and day 12. A possible explanation is that the fish were not fed anything for a few days following the treatment. With no new nutrients added to the gastrointestinal system, there could be less competition for the absorption surface or transport mechanism in the small intestine, leading to an increase in the amount of drug absorbed and transported to the liver. Another possible explanation could be an increase in the relative concentration of diflubenzuron in liver tissue due to fasting, as no other nutrients were absorbed and transported to the liver at this time, without any change in the absolute amount of diflubenzuron.

4.4 Accumulation in colon

While it is regrettable that the analytical series of colon samples taken during the treatment period did not yield reliable results, diflubenzuron is well known to have a low bioavailability and accumulate in faeces in other species, including Atlantic salmon (Horsberg and Høy, 1991), and exact measurements of drug concentration in the colon during treatment are of limited interest in terms of consumer safety. The relatively low concentrations obtained in fillet and skin and liver tissues compared to the tissue levels obtained in Atlantic salmon after the same standard treatment (see Table 4.1) indicate that accumulation in faeces occurs to an even greater extent in Atlantic cod compared to Atlantic salmon. Because many colon samples hardly contained any faecal matter, it was not possible to analyze faecal matter alone, and the presence of colon tissue in the weighed in samples leads to a lower measured concentration compared to the actual concentration in faecal matter.

On day 18, the highest calculated concentrations are 23 542, 19 163 and 11 429 ng/g (Table 3.3), which is equivalent to 23.5, 19.1 and 11.4 g/kg, respectively. Comparing this to the original in-feed concentration of diflubenzuron, which was 0.6 g/kg, diflubenzuron appears to have been concentrated up to 40 times in the colon of Atlantic cod. However, Figure 3.4 shows that the median diflubenzuron MS response in colon samples on each day during the medication period was more than twice that of the highest sample on day 18. This could be

expected, as the day 18 samples were taken 4 days after the last administered dose, and shows that the diflubenzuron accumulation in the colon during the treatment period probably far exceeded the measured concentrations on day 18.

There is a higher uncertainty associated with the colon samples compared to samples from the other matrices, as described in Section 4.1, and this is amplified by the inherent uncertainty associated with values far exceeding the 75 ng/g limit of the accredited linear range of the standard curve. Due to this, the highest concentrations in colon samples should be regarded as rough estimates rather than accurate measurements.

4.5 Excretion

All bile samples are negative for diflubenzuron (see Table 3.5). This is surprising, considering that the biliary route has been suggested as the main route of excretion for diflubenzuron in several species (IPCS, 1996). Enterohepatic circulation of diflubenzuron has been demonstrated in several other species. In Atlantic salmon, high levels of radioactivity were found in the bile after oral administration of ^{14}C-labelled diflubenzuron, and 6 hours after administration 39 % of the radioactivity was confirmed by thin layer chromatography to originate from the parent compound (Horsberg and Høy, 1991). In Sprague-Dawley rats, unconjugated diflubenzuron was found to represented about 7 % of the radiolabel in bile samples 24 hours after administration of the radiolabeled parent compound (JMPR, 2001).

From the data in Table 3.3, it is clear that all colon samples are below LOD level at day 29, which is 15 days after the last administered diflubenzuron dose. The gastrointestinal transit time of rainbow trout weighing about 80 g at 9 °C has been found to average at 68 hours, and for rainbow trout weighing about 140 g the mean transit time was 56 hours at 10 °C (Fauconneau *et al.*, 1983). As rainbow trout and Atlantic cod are both carnivores, with comparable gastrointestinal tract physiology, it is probably accurate to assume a gastrointestinal transit time of approximately 3 days for the Atlantic cod in this study, with a mean weight of 104 g and being held at 7.7 °C. This means that if the oral dose is the only route by which diflubenzuron enters the colon, most of the ingested diflubenzuron should be evacuated from the colon by day 4. Looking at Figure 3.3 and the data set in Appendix 1, there is only one negative sample on day 18, 4 days after the last oral dose, and furthermore

several of the samples on this date have a very high diflubenzuron concentration. Therefore it is probable that diflubenzuron is reentering the colon through excretion via the biliary route.

The lack of diflubenzuron response in the bile samples could be due to complex formation with insoluble bile salts, or due to metabolism of the parent compound. Another possibility is poor MS detection due to the presence of impurities in the sample which could mask the diflubenzuron signal.

Conjugation of the active substance with glucuronic acid could explain the failure to detect the parent compound in bile, while still detecting high levels in colon. Bacteria in the colon produce enzymes with beta-glucuronidase activity, which hydrolyze the glucuronide conjugate back to glucuronic acid and free parent compound. However, diflubenzuron is hydroxylated prior to glucuronide conjugation. The major metabolic pathway of diflubenzuron in rats and cows, as illustrated in Figure 1.3, is the hydroxylation of the parent compound to 4-chloro-2-hydroxydiflubenzuron, 4-chloro-3-hydroxydiflubenzuron, and 2,6-difluoro-3-hydroxydiflubenzuron. These metabolites can be conjugated with glucuronic acid and excreted via bile, but hydrolysis catalyzed by bacterial enzymes in the colon will regenerate the hydroxylated metabolite of diflubenzuron and not the parent compound.

4.6 Consumer safety

At no time during the treatment did any of the fillet and skin samples exceed the MRL value of 1000 ng/g. Furthermore, the likelihood that any wild fish outside the pens should consume an amount of medicated feed equivalent to or exceeding the standard treatment dose is low.

p-Chloroaniline was not detected in any of the fillet and skin samples from the treatment period, with a LOD of 2 ng/g.

In this study, the median concentration obtained in Atlantic cod fillet and skin during the treatment period was 36.1 ng/g, and the LOD is 5.5 % of this concentration. Based on data from pigs (Opdycke *et al.*, 1982) and rats (IPCS, 1996), in which about 0.06 and 0.01 %, respectively, of the absorbed diflubenzuron dose has been found to be converted to PCA , much less than 1 % of the absorbed diflubenzuron dose is likely to be converted to PCA in Atlantic cod. Due to the low obtained median concentration of diflubenzuron, the potential PCA concentration would most likely be undetectable. This means that PCA cannot be ruled

out as a metabolite of diflubenzuron in Atlantic cod based on the data from this study, but even though the metabolic pathway could be present in Atlantic cod, the potential PCA concentration would be extremely low due to the low gastrointestinal diflubenzuron uptake of Atlantic cod.

Conclusion

The median concentration obtained in samples of fillet and skin throughout the treatment period was 36.1 ng/g, which is only 1.5 % of the mean concentration obtained in Atlantic salmon fillet after the same treatment. This shows that diflubenzuron probably has a lower gastrointestinal uptake in Atlantic cod compared to Atlantic salmon. Higher concentrations are however recorded in some fillet and skin samples from the treatment period (329.1, 633.3 and 744.8 ng/g), and it is possible that these extreme values come from dominant individuals who have consumed a relatively high proportion of the medicated feed. There was a high variability in the obtained results, this may in part be caused by dominant individuals, but it could also be due to a high variation in the day-to-day food intake of Atlantic cod. It seems that voluntary oral administration may be a poor administration route for substances with a low gastrointestinal uptake in Atlantic cod due to its feeding behaviour.

A comparison of tissue levels of diflubenzuron in Atlantic salmon and Atlantic cod shows that diflubenzuron is unlikely to be effective against louse infection in Atlantic cod because the obtained median steady state concentration in fillet and skin during the treatment (sample days 4-15) is only 36.1 ng/g, compared to the minimum effective tissue concentration of 900 ng/g that is assumed by the industry, and the mean steady state levels of 2240 and 1550 ng/g that have been obtained in fillet and skin samples of Atlantic salmon at 6 and 15 °C, respectively.

para-Chloroaniline was not detected in samples of fillet and skin from the treatment period at a LOD level of 2 ng/g. This however does not rule out the formation of this metabolite in Atlantic cod, because the tissue level of diflubenzuron was so low that the fraction of PCA which may be formed would most likely be undetectable. In terms of consumer safety however, there does not seem to be any risk of exposure to PCA from consumption of wild caught Atlantic cod which may have fed on medicated feed spills from salmon farming facilities.

Proposal for further studies

While the analytical method was satisfactory for samples of cod fillet and skin in natural proportions, problems were encountered in the purification and quantification steps for the other matrices, colon and bile samples in particular. The choice of solvents for extraction clearly is not ideal for these matrices, and optimal purification of the samples was not achieved. This in turn resulted in a high noise level in the MS spectra, making the quantification of diflubenzuron difficult in these samples.

In the study by Horsberg and Høy (1991), purification of bile samples was achieved through 1:5 dilution with methanol in order to precipitate mucus and proteins, and subsequent centrifugation before the supernatant was collected. This purification method may be more suitable prior to HPLC/MS analysis of bile samples.

In order to accurately determine the gastrointestinal uptake of diflubenzuron in Atlantic cod, a study design applying oral administration by gavage is probably more appropriate, due to the periodical feeding and territorial behavior of Atlantic cod.

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Appendix

Fillet and skin samples							
Sample	**DIF (ng/g)**	**Area DIF**	**Area IS**	**Sample**	**DIF (ng/g)**	**Area DIF**	**Area IS**
110	0	10929	186094	**155**	0	0	112294
111	329.1	1610144	206829	**156**	0	7738	289896
112	15.2	58273	162555	**157**	46.5	181248	164789
113	77.8	384731	208968	**158**	0	7425	157461
114	153.0	795752	219886	**159**	0	0	152411
115	44.2	192287	184010	**160**	0	0	284980
116	50.3	376786	316888	**161**	0	0	217646
117	10.0	27712	178563	**162**	0	0	243895
118	10.0	27937	210378	**163**	0	0	305258
119	10.8	43977	171695	**164**	0	0	283249
120	0	8402	169339	**165**	0	0	219285
121	10.0	47056	223714	**166**	0	0	240480
122	0	18874	238268	**167**	0	0	177659
123	19.8	99848	213705	**168**	0	0	284471
124	10.0	51332	247171	**169**	0	0	223791
125	15.6	70111	189633	**170**	0	0	245084
126	58.8	298028	214189	**171**	0	0	208787
127	73.4	422658	243318	**172**	0	0	267884
128	10.0	36891	192728	**173**	0	0	207009
129	14.1	72457	216570	**174**	0	0	226612
130	221.1	1248541	238781	**175**	0	0	203527
131	45.3	243553	227286	**176**	0	0	269097
132	13.1	60310	194339	**177**	0	0	217839
133	0	10136	187424	**178**	0	0	201428
134	633.3	3460357	230987	**179**	0	0	188865
135	18.6	95384	216416	**180**	0	0	218508
136	48.6	218344	189784	**181**	0	0	156552
137	49.9	211247	178945	**182**	0	0	192565
138	74.3	526259	299580	**183**	0	0	195617
139	138.8	668911	203708	**184**	0	0	197518
140	86.0	528316	259832	**185**	0	0	170588
141	35.0	194296	234922	**186**	0	0	169351
142	37.2	164955	187245	**187**	0	0	193984
143	10.0	39658	183763	**188**	0	0	185930
144	10.4	51257	208686	**189**	0	0	180498
145	110.2	611494	234573	**190**	0	0	199874
146	744.8	2460284	139650	**191**	0	0	197845
147	83.7	463048	233929	**192**	0	0	207492
148	33.0	155623	199541	**193**	0	0	211689
149	0	15393	231261	**194**	0	0	167200
150	0	15941	154561	**195**	0	0	182061
151	0	5462	203580	**196**	0	0	234236
152	0	6947	216143	**197**	0	0	208671
153	0	0	176883	**198**	0	0	204231
154	0	0	156420	**199**	0	0	145382

Liver samples							
Sample	**DIF (ng/g)**	**Area DIF**	**Area IS**	**Sample**	**DIF (ng/g)**	**Area DIF**	**Area IS**
110	19.0	27224	79496	**155**	0	2164	76562
111	86.4	121226	77664	**156**	0	0	76247
112	124.8	169963	75481	**157**	0	0	71970
113	457.4	424208	51385	**158**	94.0	63580	67817
114	113.4	162634	79471	**159**	0	0	65896
115	77.4	109462	78378	**160**	0	0	70521
116	72.2	106646	81731	**161**	0	0	67919
117	19.6	11980	33949	**162**	0	0	69045
118	75.2	106743	78557	**163**	0	0	69025
119	95.6	136452	79047	**164**	0	0	39633
120	53.8	74643	76850	**165**	0	0	66894
121	148.8	135404	50411	**166**	0	0	70807
122	72.4	91309	69950	**167**	0	0	65030
123	48.2	66599	76559	**168**	0	0	74537
124	50.6	70035	76820	**169**	16.8	24711	81544
125	-	-	8604	**170**	0	0	79430
126	106.0	143499	75023	**171**	0	0	84702
127	163.6	207108	70121	**172**	0	0	78173
128	152.8	207956	75392	**173**	0	0	88315
129	68.6	38714	31290	**174**	0	0	79731
130	14.6	19262	72993	**175**	0	0	82676
131	100.6	137250	75590	**176**	0	0	78384
132	27.0	35190	72414	**177**	0	0	87327
133	0	1934	24593	**178**	0	0	80418
134	122.8	156664	70635	**179**	0	0	84932
135	128.4	171746	74136	**180**	0	0	70866
136	63.4	81427	71150	**181**	0	0	74556
137	191.2	119979	34776	**182**	0	0	83666
138	51.2	66979	72423	**183**	0	0	72800
139	49.6	57128	63782	**184**	0	0	82229
140	111.4	160150	144171	**185**	0	0	76880
141	186.8	251065	134790	**186**	0	0	76313
142	111.4	151268	136203	**187**	0	0	73689
143	132.0	177097	134471	**188**	0	0	82291
144	178.6	214166	120246	**189**	0	0	70866
145	274.6	312662	114132	**190**	0	0	81332
146	308.4	361757	117576	**191**	0	0	80181
147	170.6	201804	118585	**192**	0	0	69477
148	214.6	204061	95340	**193**	0	0	84401
149	124.6	116813	94063	**194**	0	0	79327
150	10.0	10411	110579	**195**	0	0	88782
151	52.8	42449	80545	**196**	0	0	77534
152	0	0	83103	**197**	0	0	86876
153	0	0	52670	**198**	0	0	85401
154	0	0	73368	**199**	0	0	82077

Colon samples							
Sample	**DIF (ng/g)**	**Area DIF**	**Area IS**	**Sample**	**DIF (ng/g)**	**Area DIF**	**Area IS**
110	-	42635440	40477	**155**	11429.0	4545397	7382
111	-	44621144	42374	**156**	0	0	57859
112	-	33914016	36655	**157**	19163.0	11474969	12828
113	-	28675238	24129	**158**	390.6	115205	5323
114	-	46021168	54207	**159**	78.6	82836	21140
115	-	23082896	41007	**160**	0	0	46754
116	-	30101072	39722	**161**	20.7	34922	71442
117	-	23321662	53867	**162**	0	0	149441
118	-	30461382	38865	**163**	0	0	110211
119	-	48901928	40208	**164**	0	0	132865
120	-	33915928	40564	**165**	0	0	24560
121	-	34211316	39927	**166**	0	0	27811
122	-	24844100	23366	**167**	0	0	8205
123	-	28239496	30328	**168**	0	0	75815
124	-	33133834	25033	**169**	0	0	22099
125	-	12621338	11864	**170**	0	0	60253
126	-	33841440	22308	**171**	0	0	53606
127	-	22944286	20306	**172**	0	0	143314
128	-	40599224	66833	**173**	0	0	131811
129	-	29312130	27834	**174**	0	0	232587
130	-	56272720	60513	**175**	0	0	42452
131	-	40065604	47906	**176**	0	0	22189
132	-	32298348	32140	**177**	0	0	23309
133	-	-	-	**178**	0	0	18659
134	-	-	-	**179**	0	0	8473
135	-	-	-	**180**	0	0	46722
136	-	-	-	**181**	0	0	63538
137	-	25154706	18535	**182**	0	0	110210
138	-	41081928	40646	**183**	0	0	261123
139	-	26345868	52240	**184**	0	0	36206
140	-	21309280	16769	**185**	0	0	13816
141	-	20945866	16752	**186**	0	0	20814
142	-	32334454	23704	**187**	0	0	51239
143	-	32494974	36049	**188**	0	0	9950
144	-	36434108	43468	**189**	0	0	28599
145	-	34801600	27304	**190**	0	0	249121
146	-	25985430	18411	**191**	0	0	277513
147	-	31386668	36691	**192**	0	0	295062
148	-	32831320	25222	**193**	0	0	80515
149	-	28675944	31735	**194**	0	0	56533
150	23542.0	15699389	19998	**195**	0	0	53907
151	407.6	395900	27491	**196**	0	0	58482
152	35.1	29558	21805	**197**	0	0	87042
153	493.7	489730	28072	**198**	0	0	41675
154	12.2	15102	28346	**199**	0	0	55823

Appendix 2 Standard curves for each analytical series

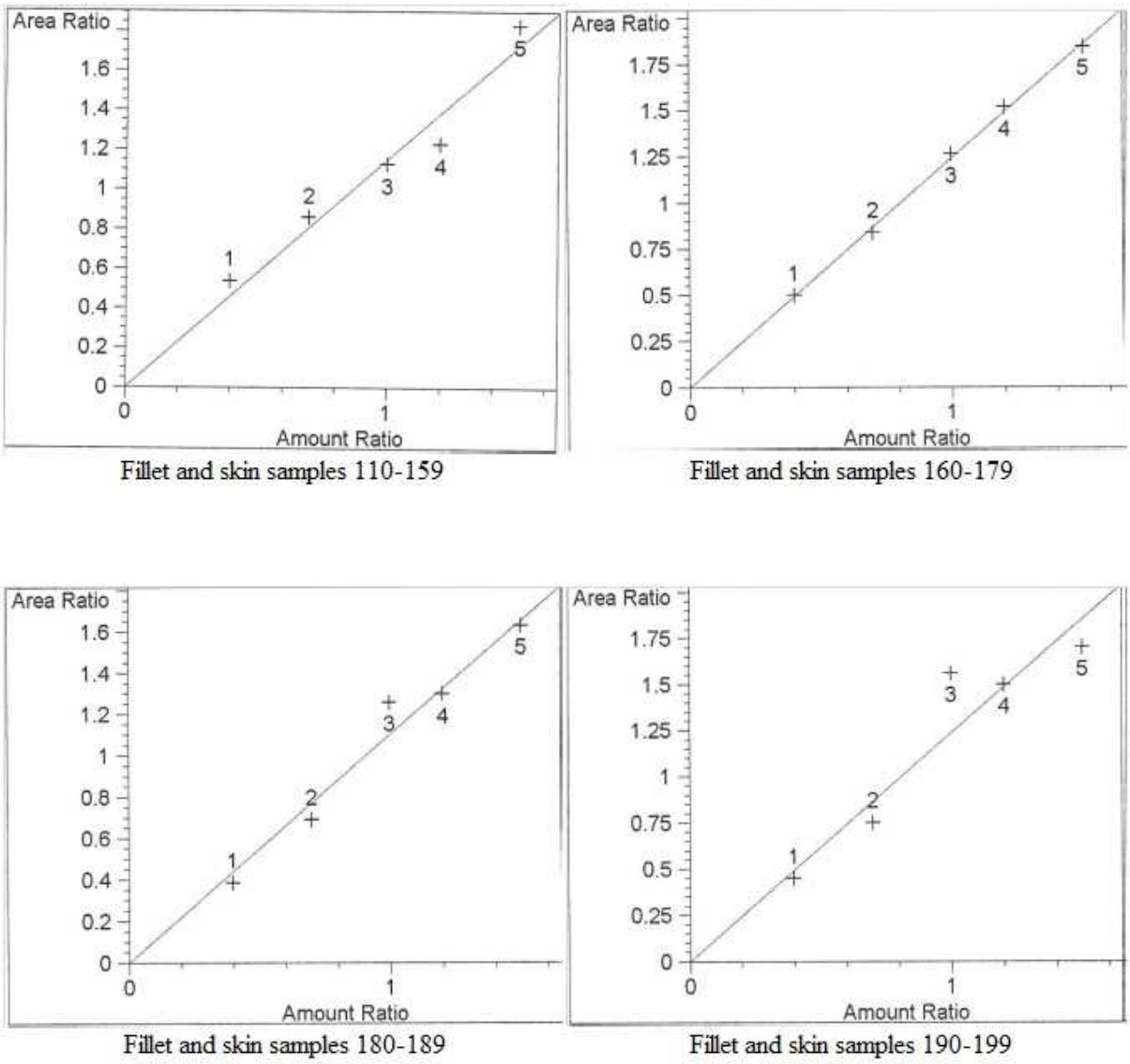
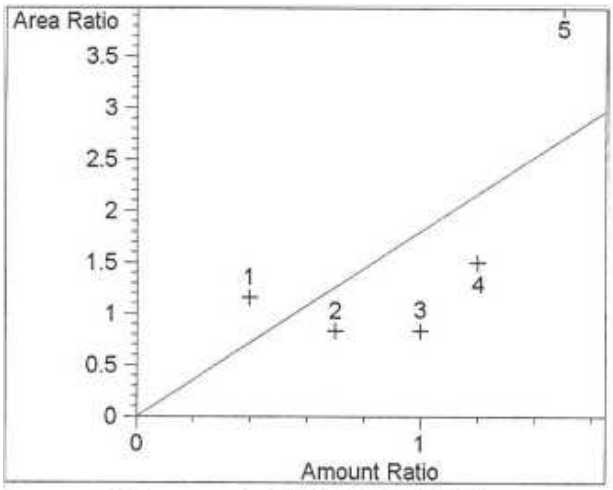
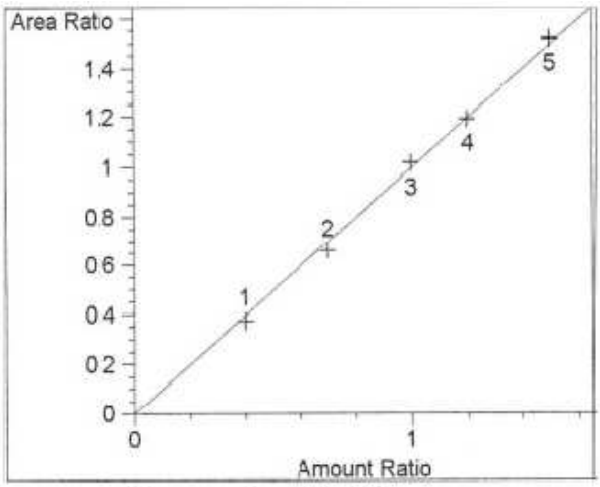


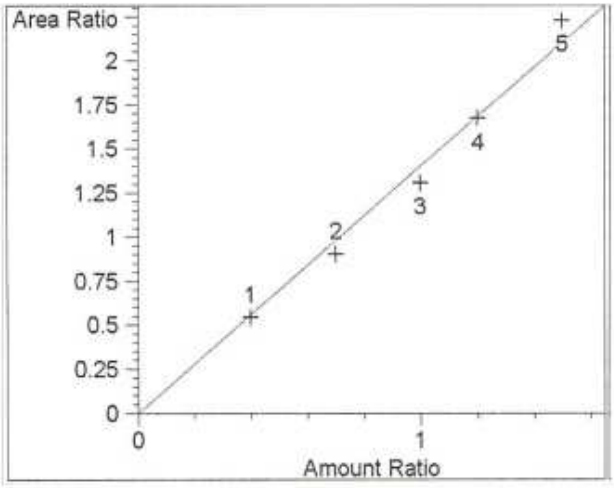
Figure A.1 Standard curves for fillet and skin series



Liver samples 110-139, 163-169

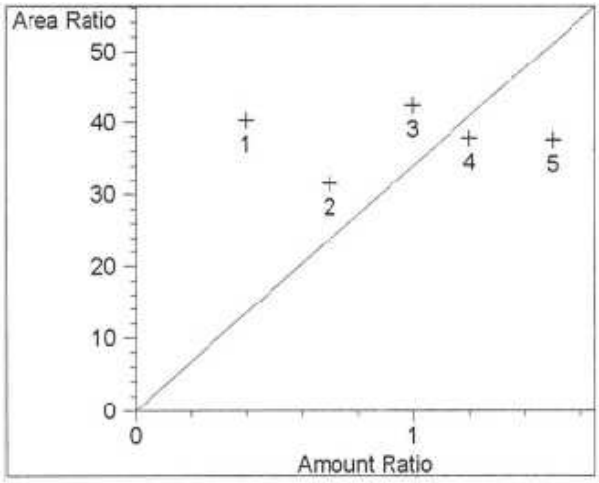


Liver samples 140-162

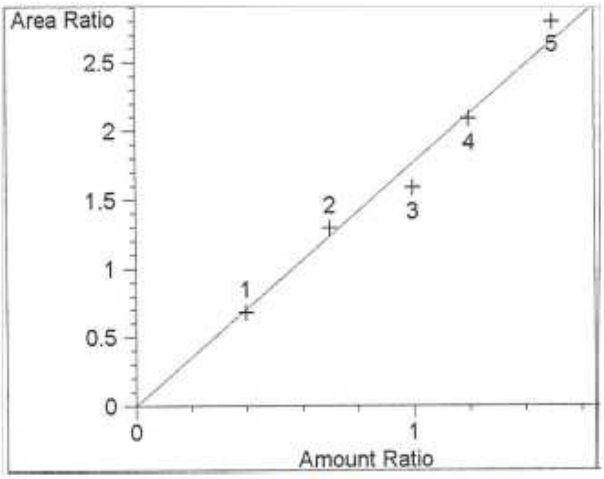


Liver samples 170-199

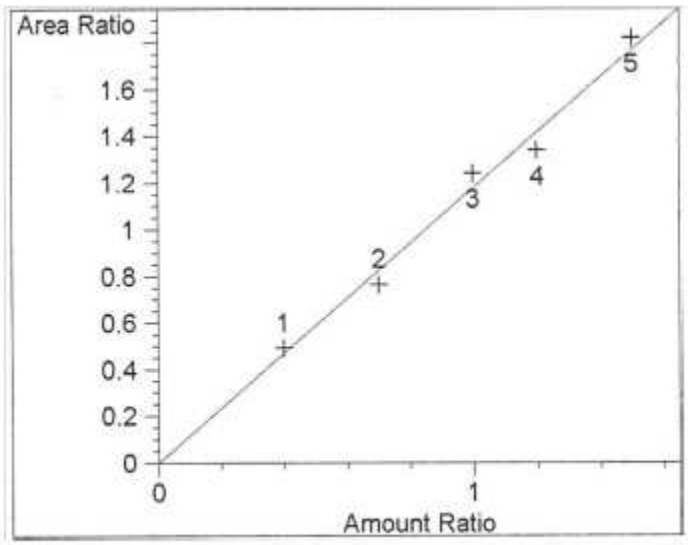
Figure A.2 Standard curves for liver series



Colon samples 110-149



Colon samples 150-159, 165-169, 175-179, 185-189, 195-199



Colon samples 160-164, 170-174, 180-184, 190-194

Figure A.3 Standard curves for colon series

Appendix 3 Sample spectra from all matrices

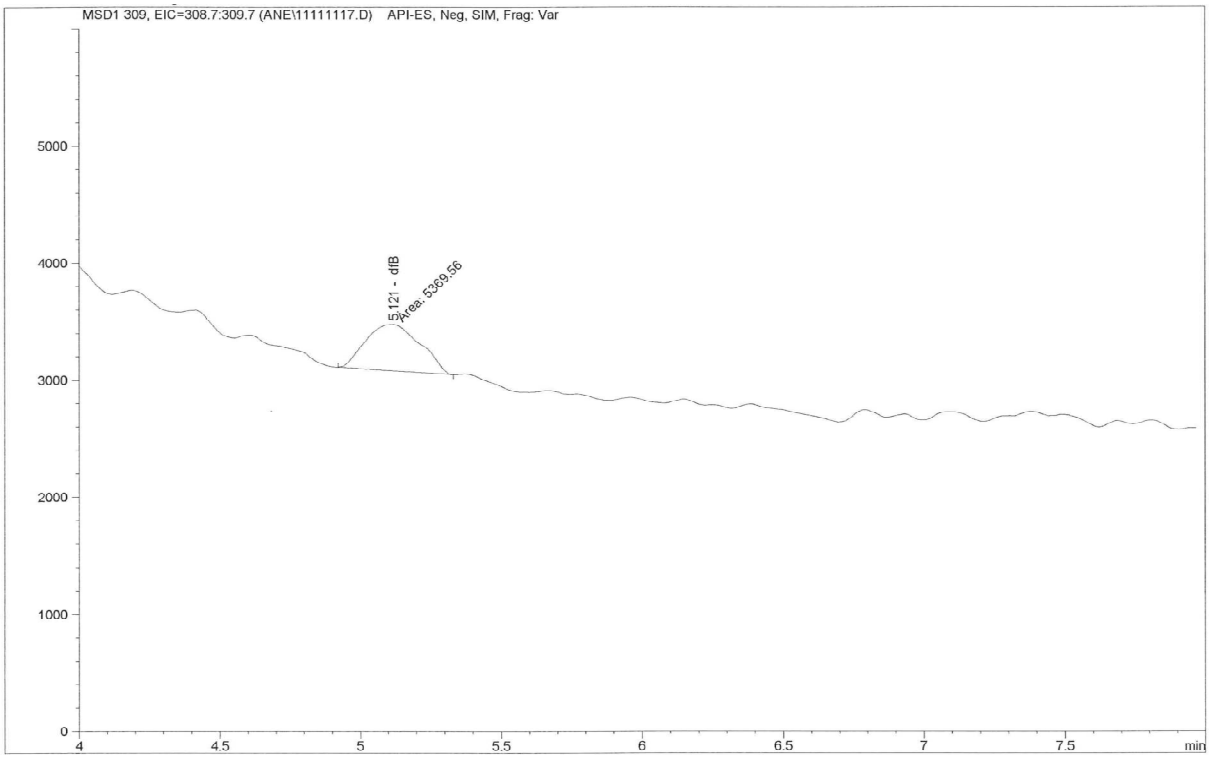


Figure A.4 Fillet and skin, sample 110

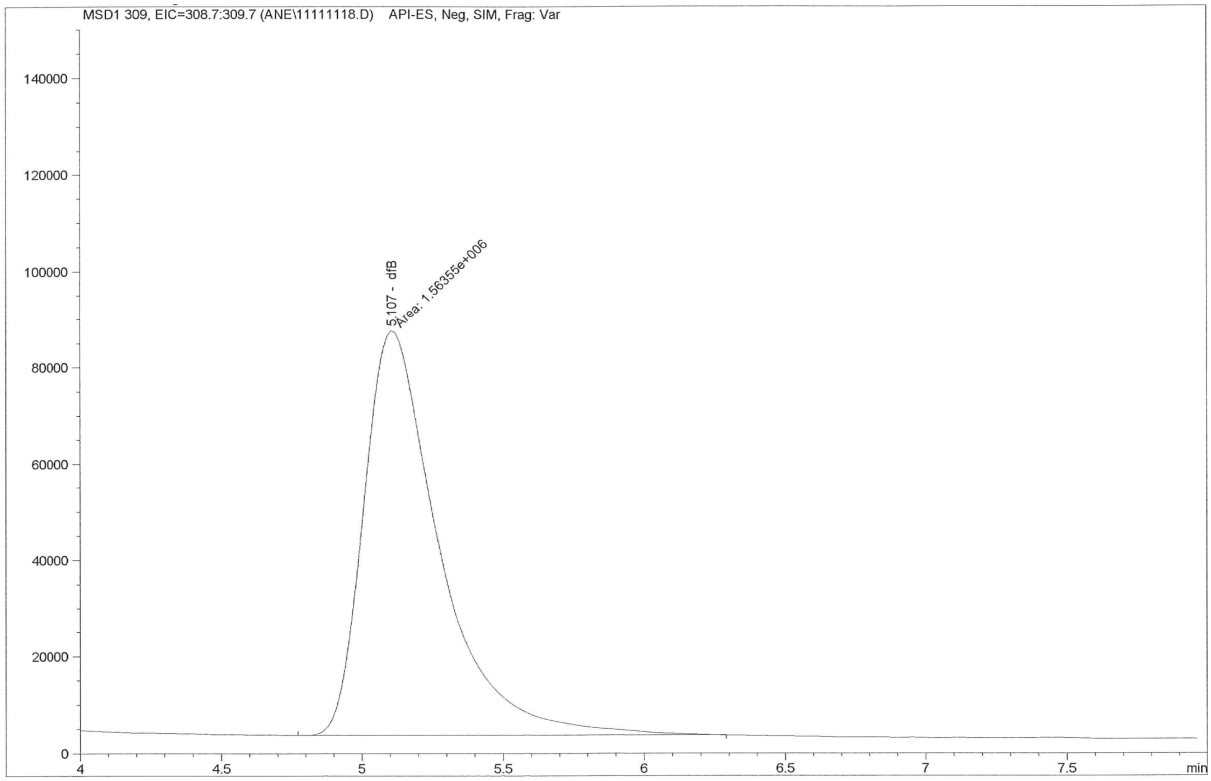


Figure A.5 Fillet and skin, sample 111

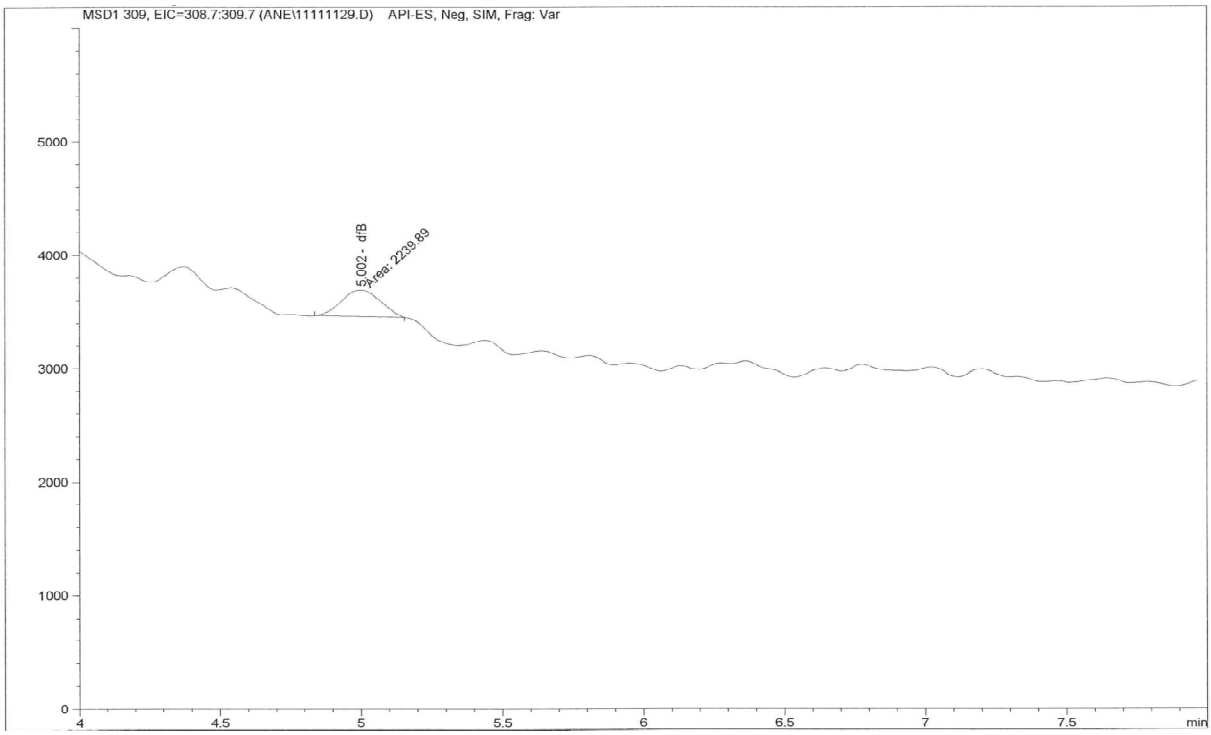


Figure A.6 Fillet and skin, sample 120

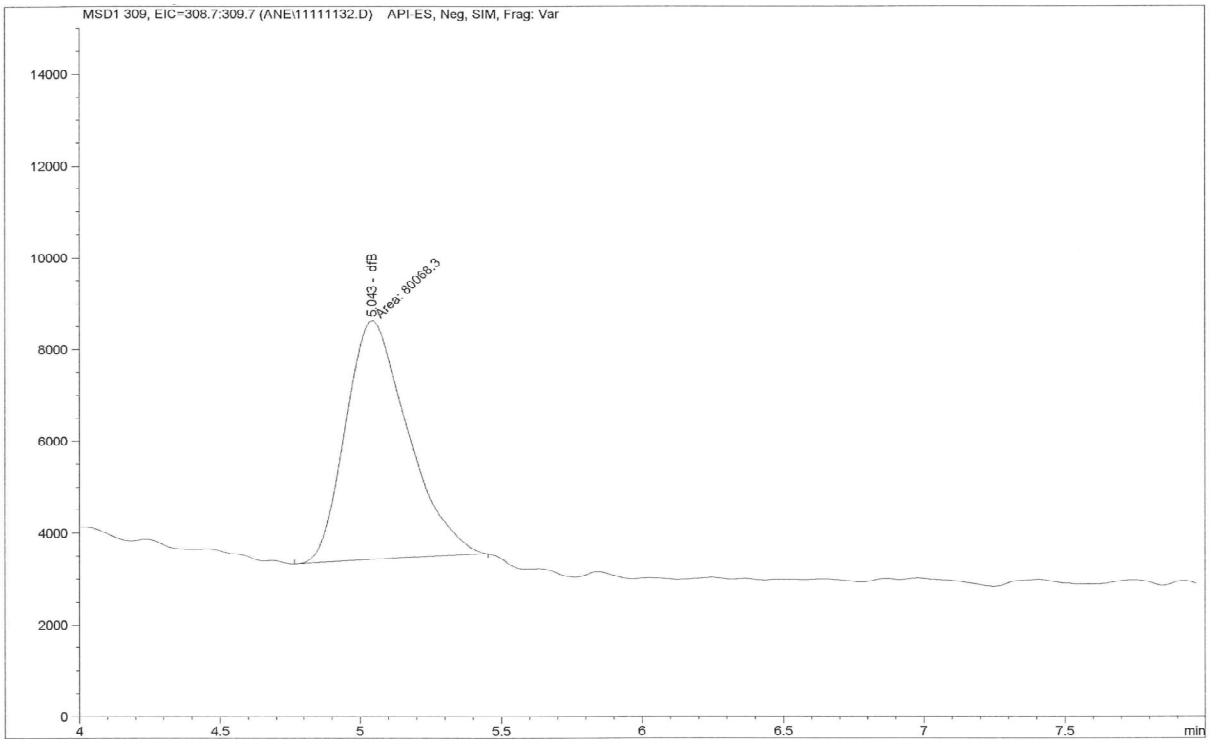


Figure A.7 Fillet and skin, sample 123

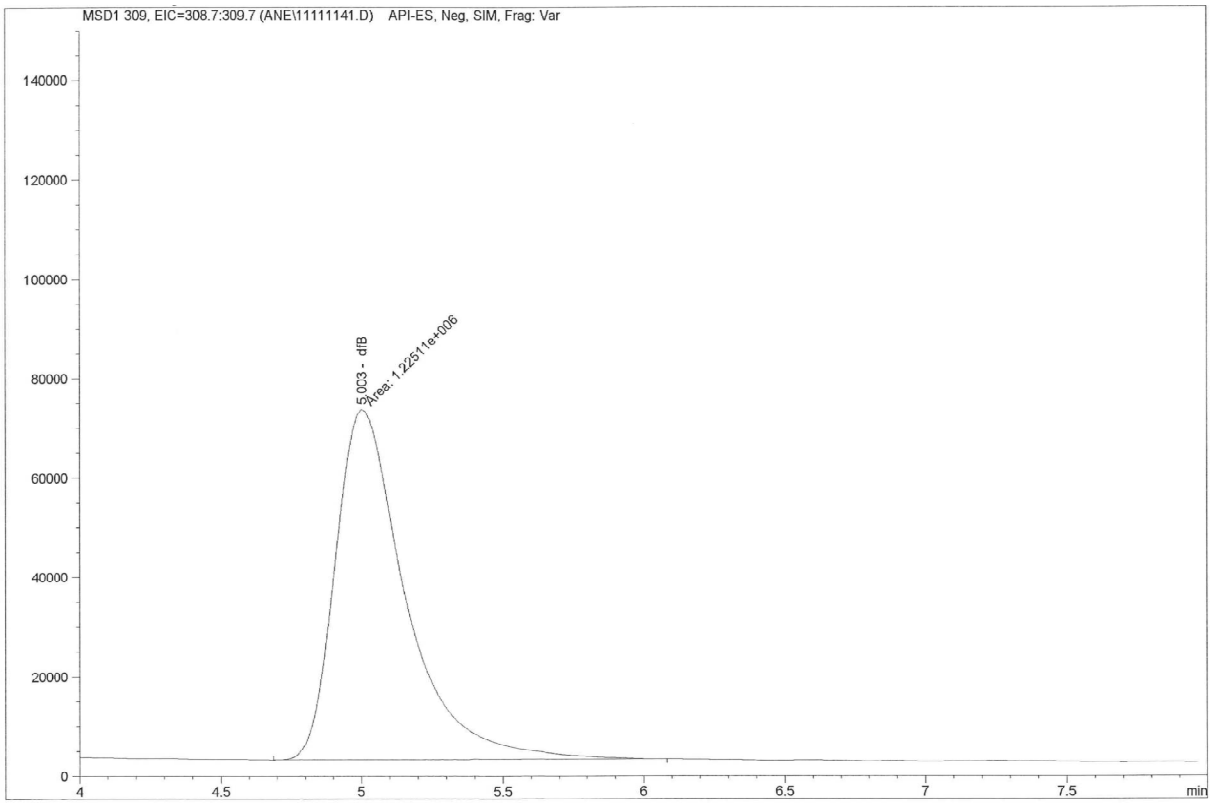


Figure A.8 Fillet and skin, sample 130

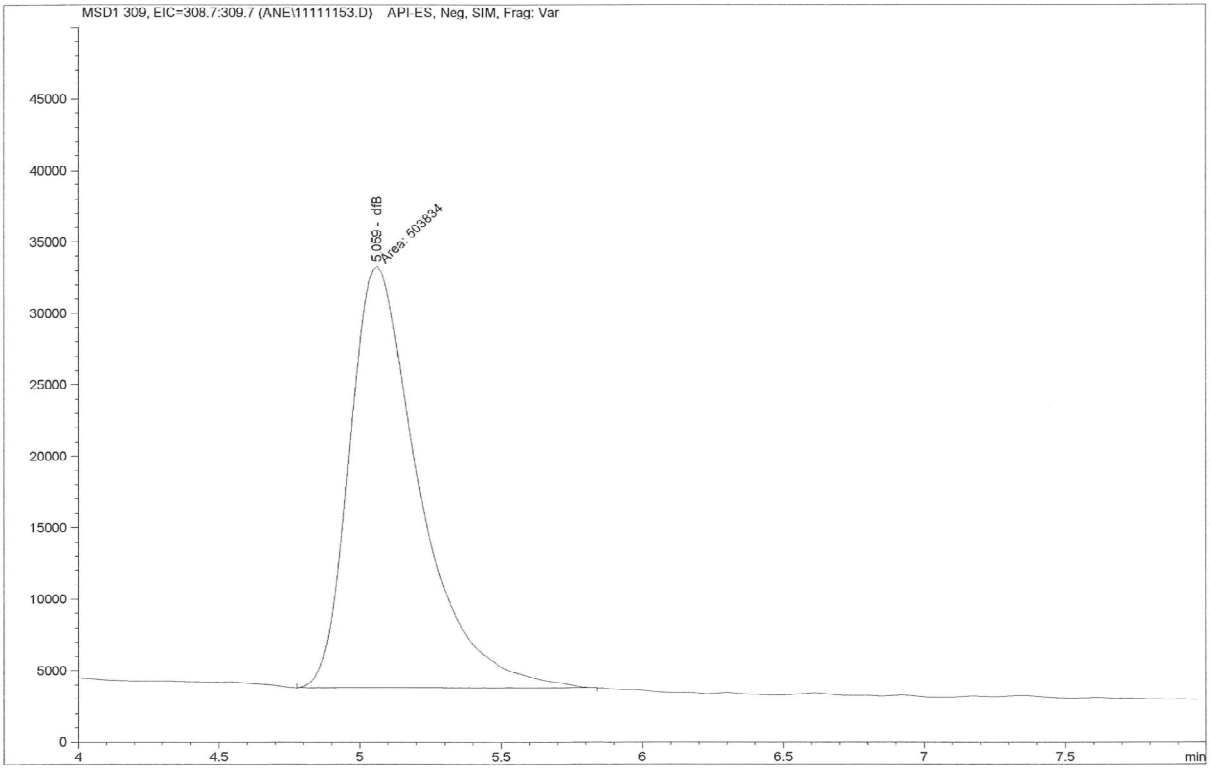


Figure A.9 Fillet and skin, sample 140

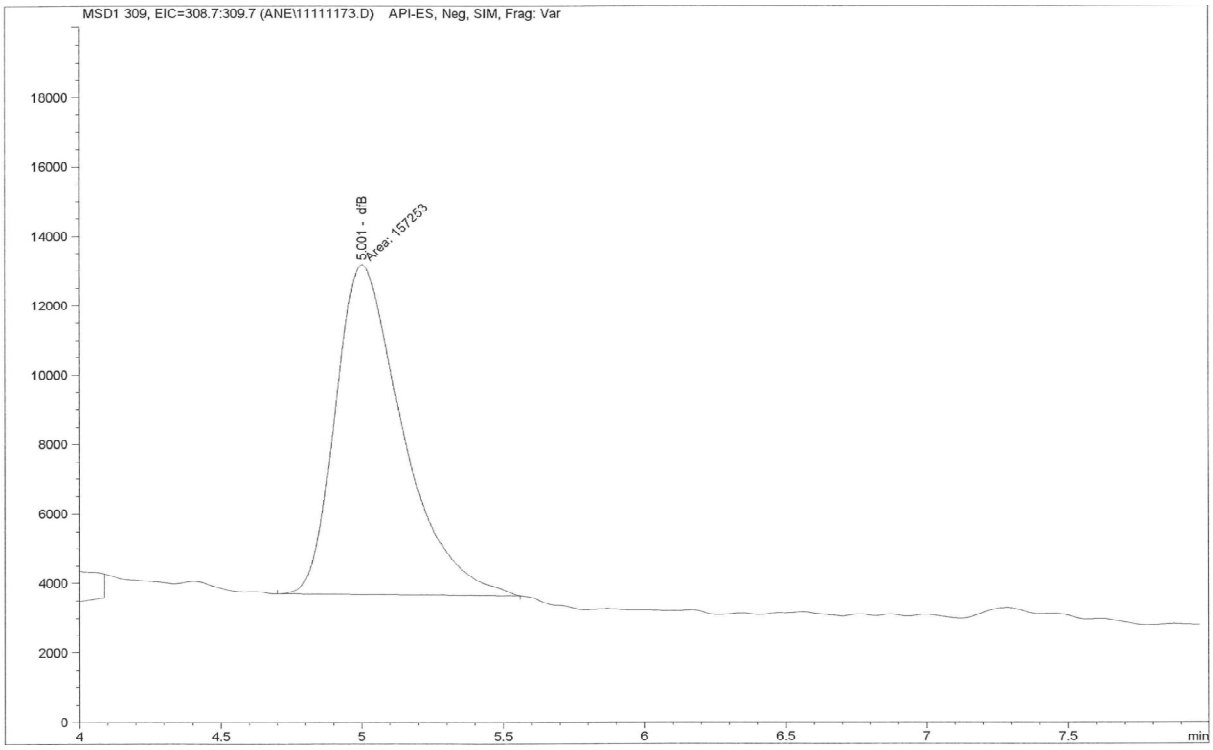


Figure A.10 Fillet and skin, sample 157

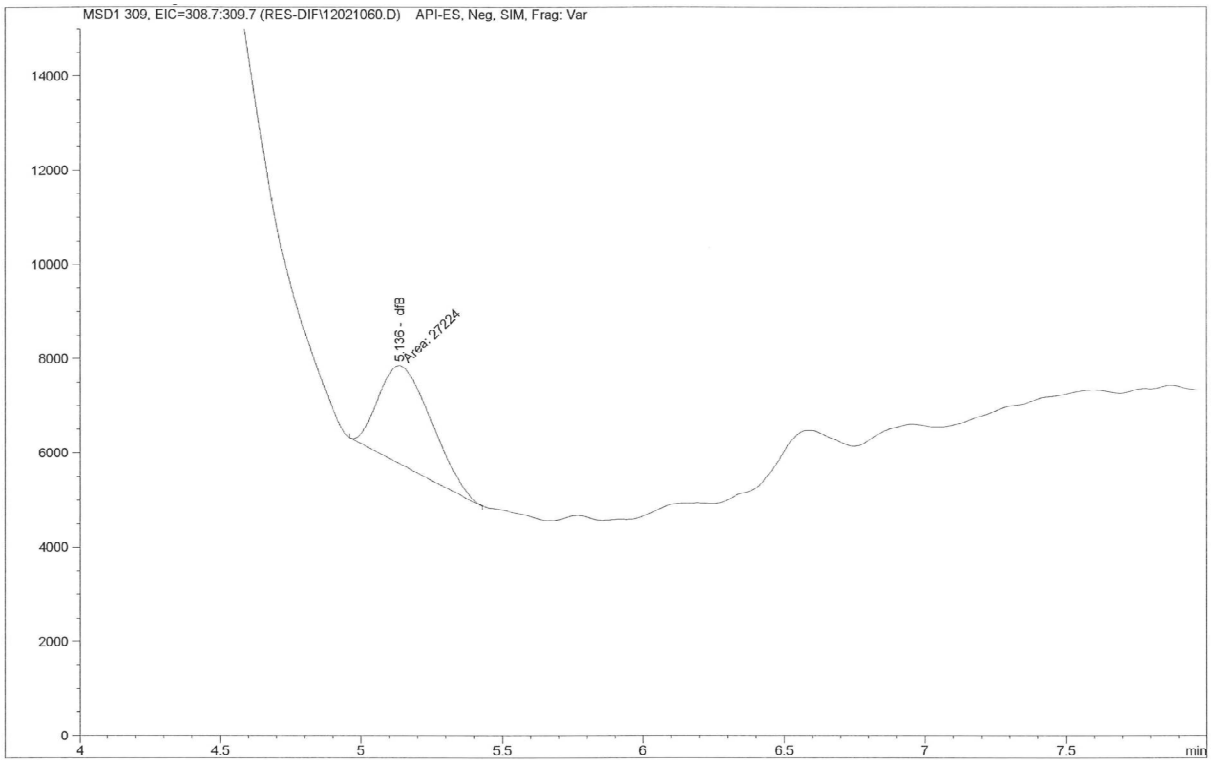


Figure A.11 Liver, sample 110

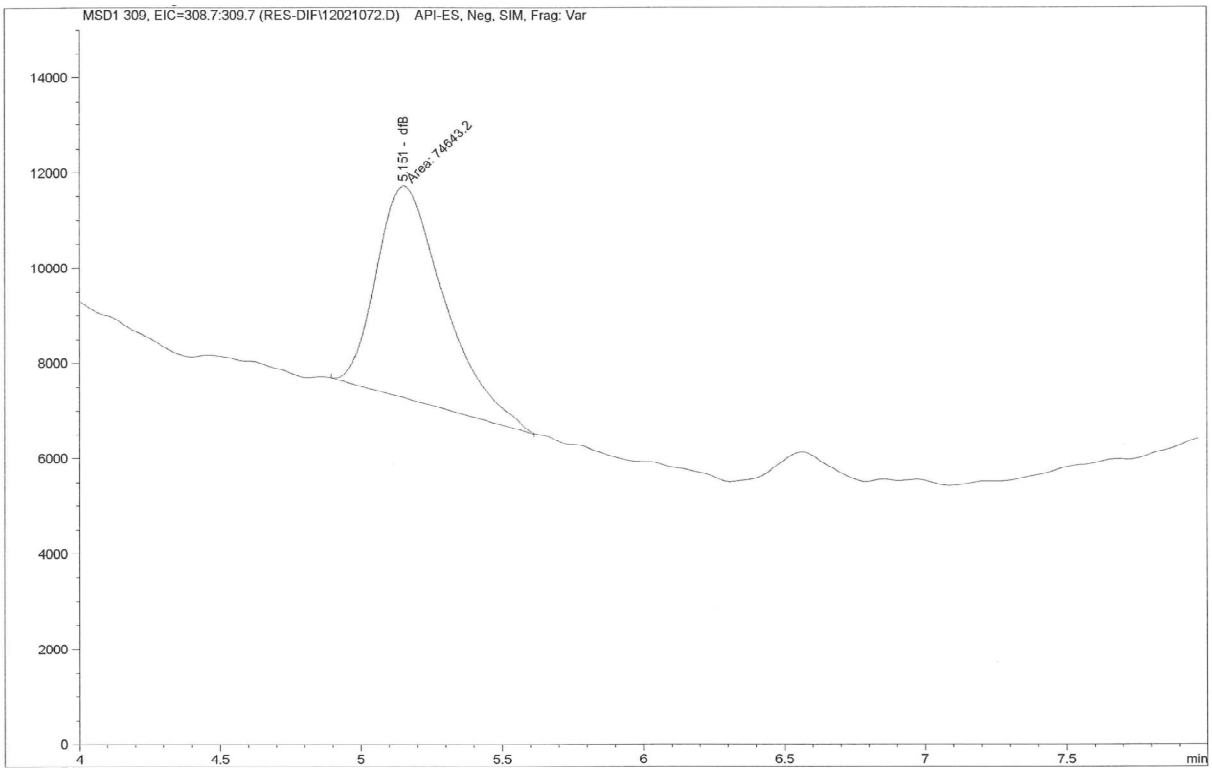


Figure A.12 Liver, sample 120

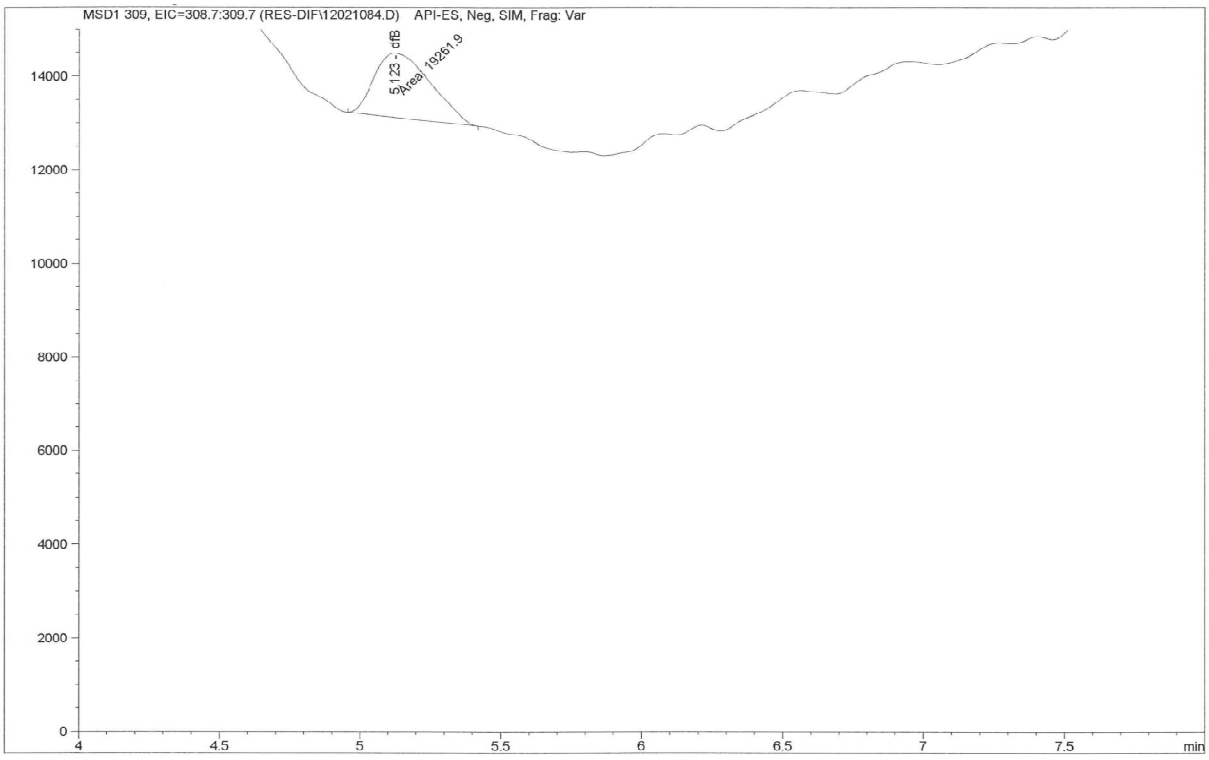


Figure A.13 Liver, sample 130

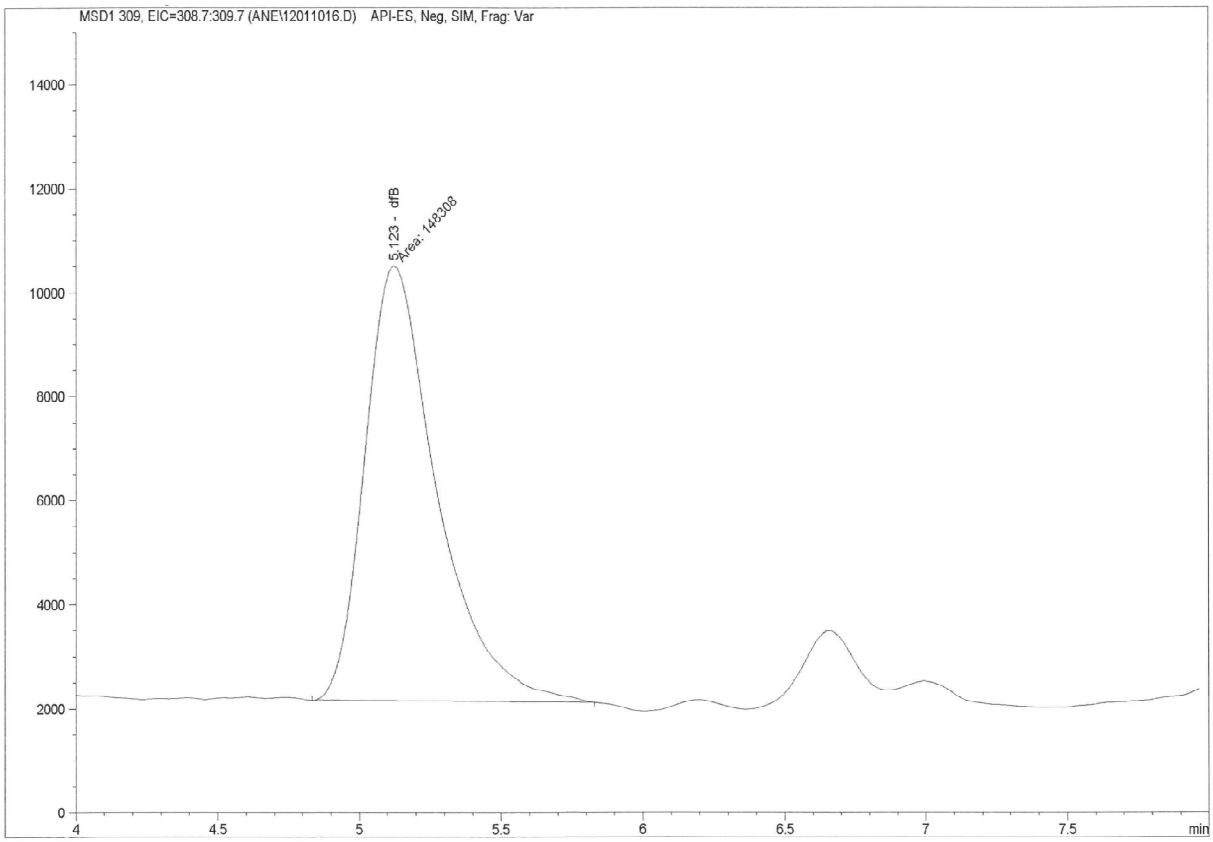


Figure A.14 Liver, sample 140

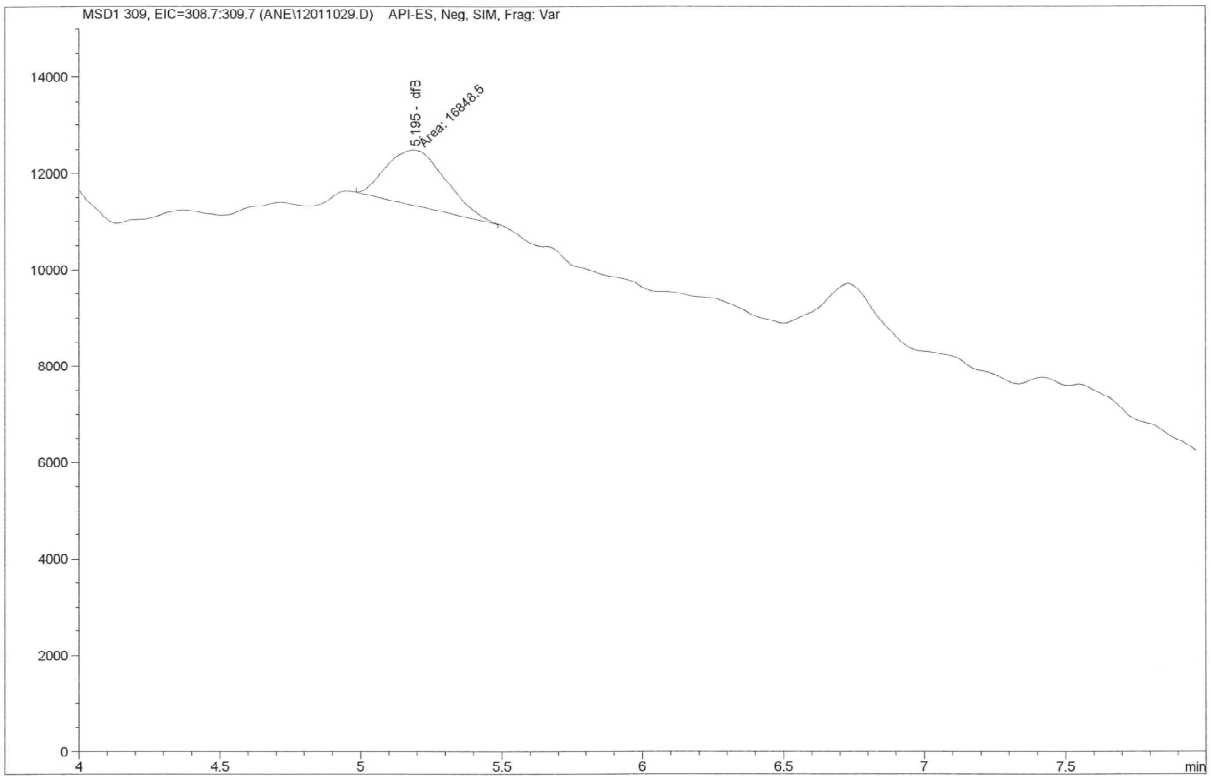


Figure A.15 Liver, sample 151

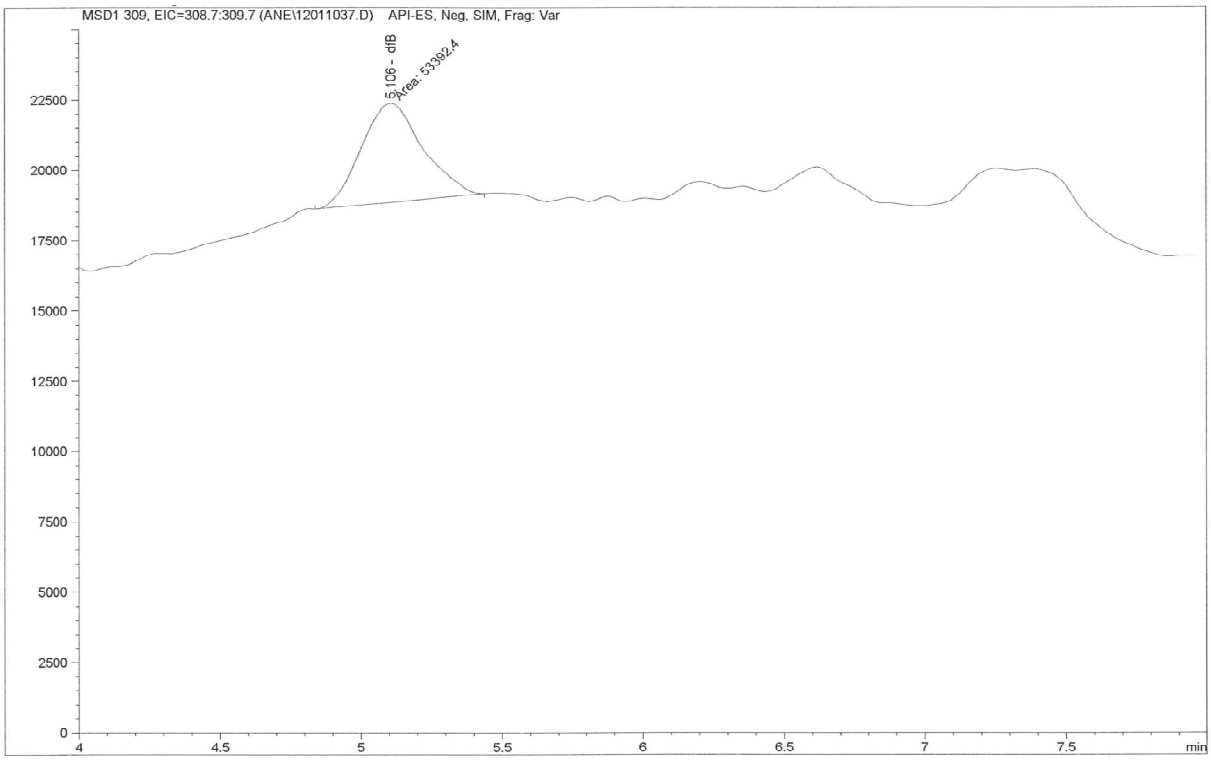


Figure A.16 Liver, sample 158

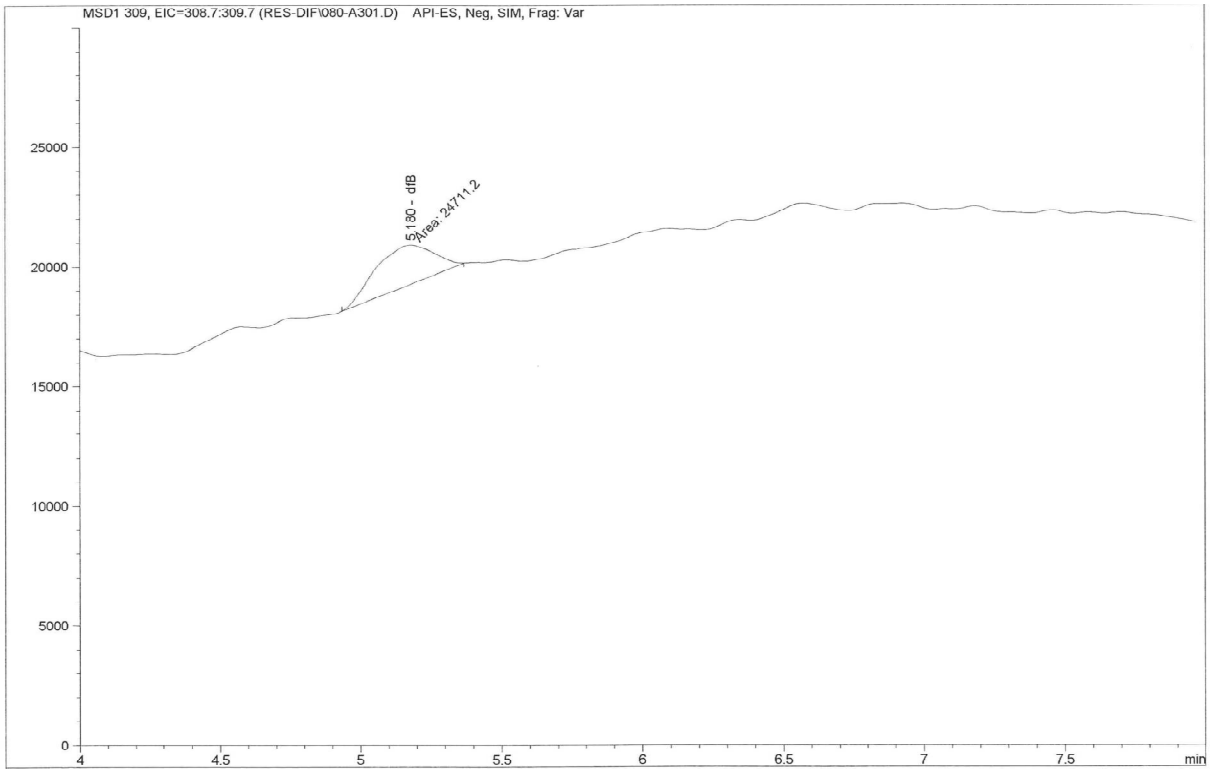


Figure A.17 Liver, sample 169

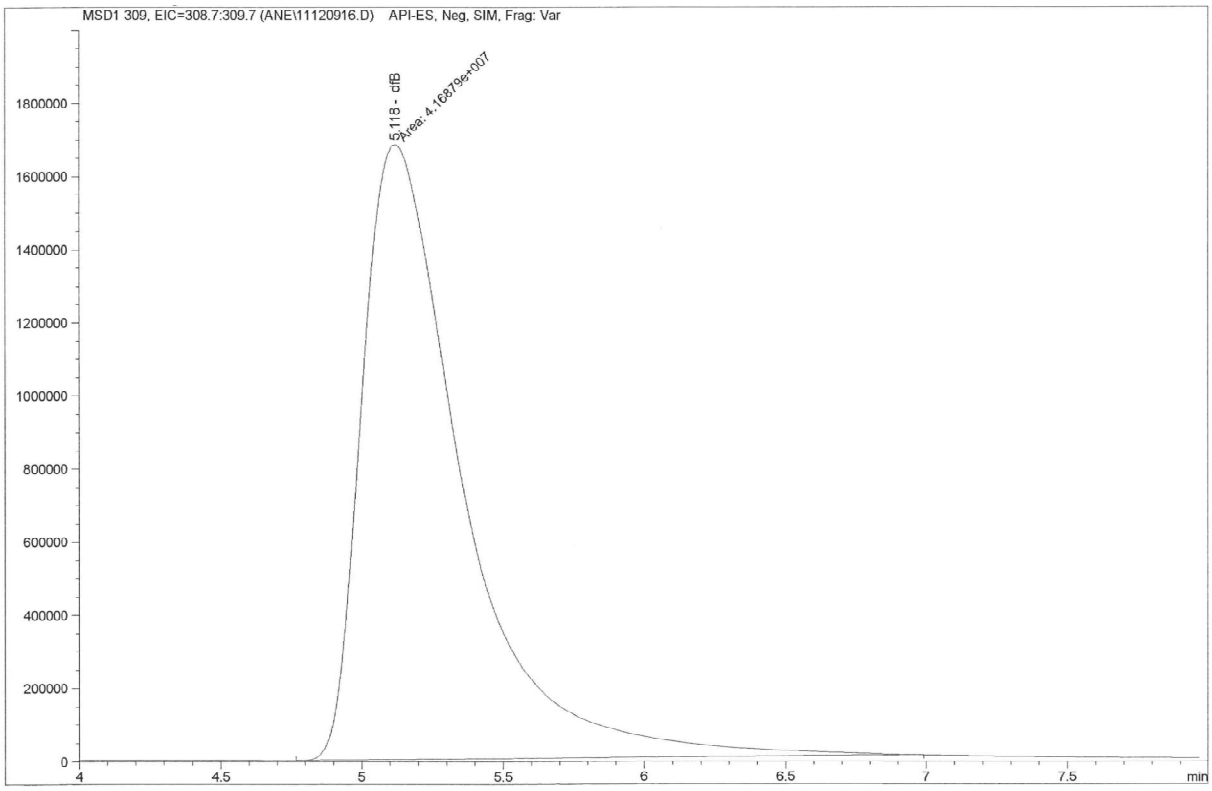


Figure A.18 Colon, sample 110

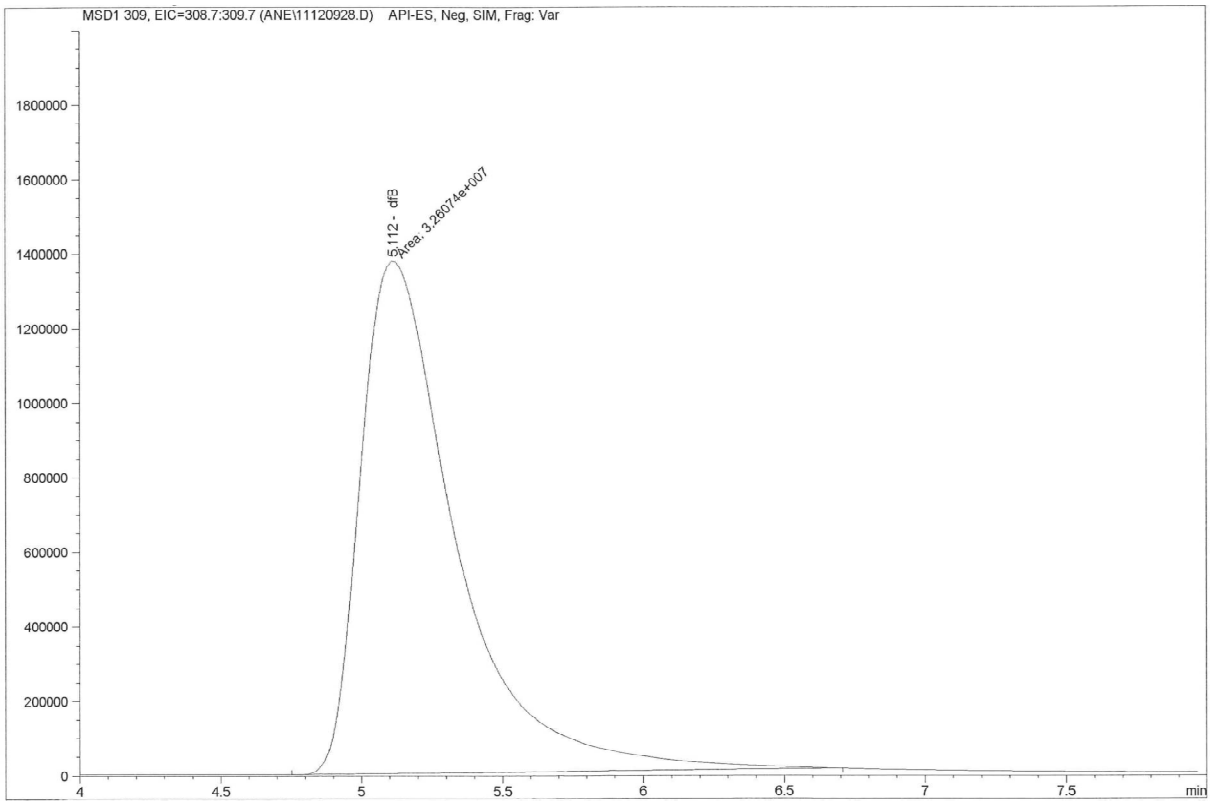


Figure A.19 Colon, sample 120

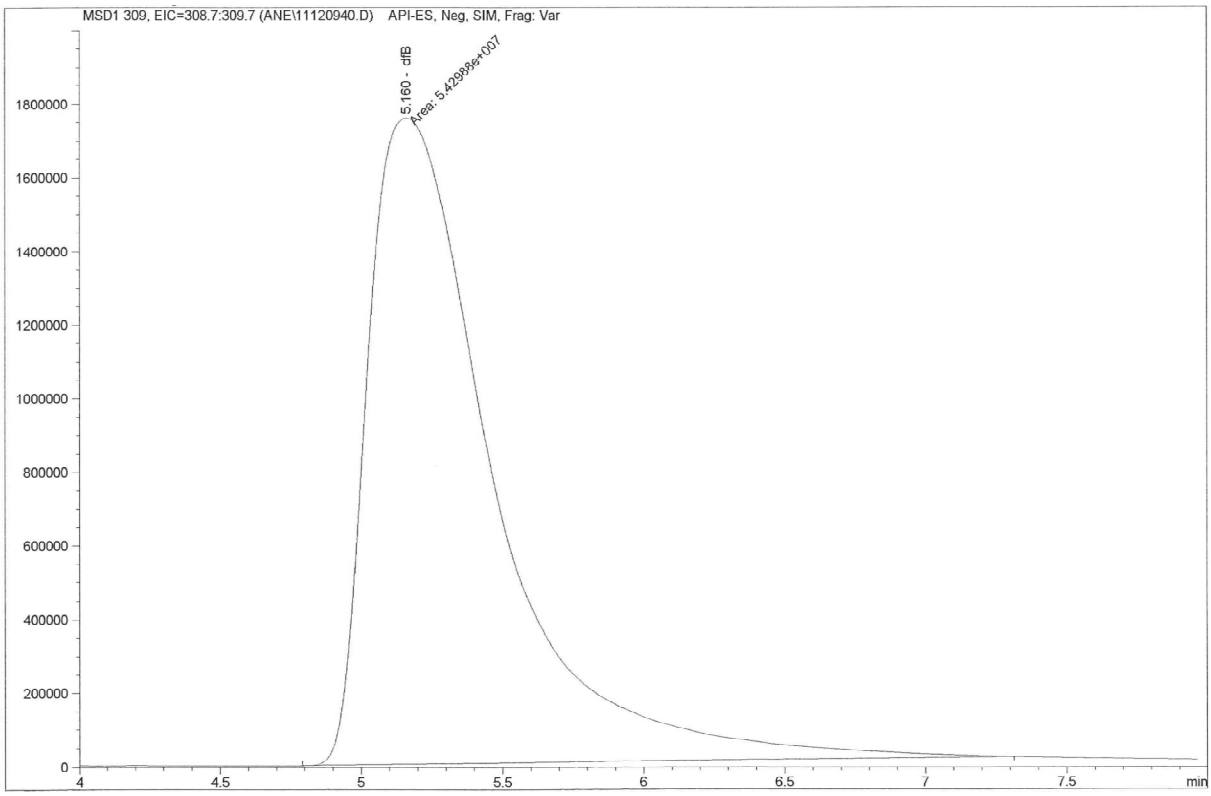


Figure A.20 Colon, sample 130

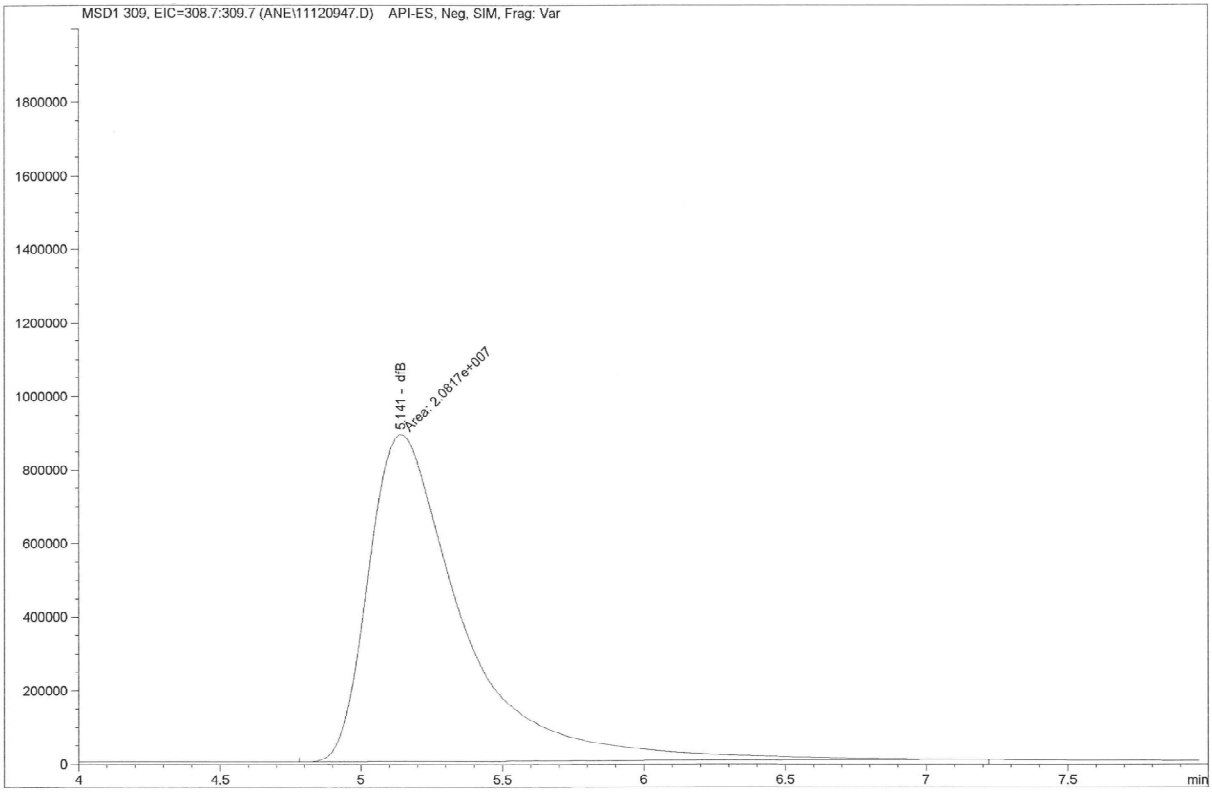


Figure A.21 Colon, sample 140

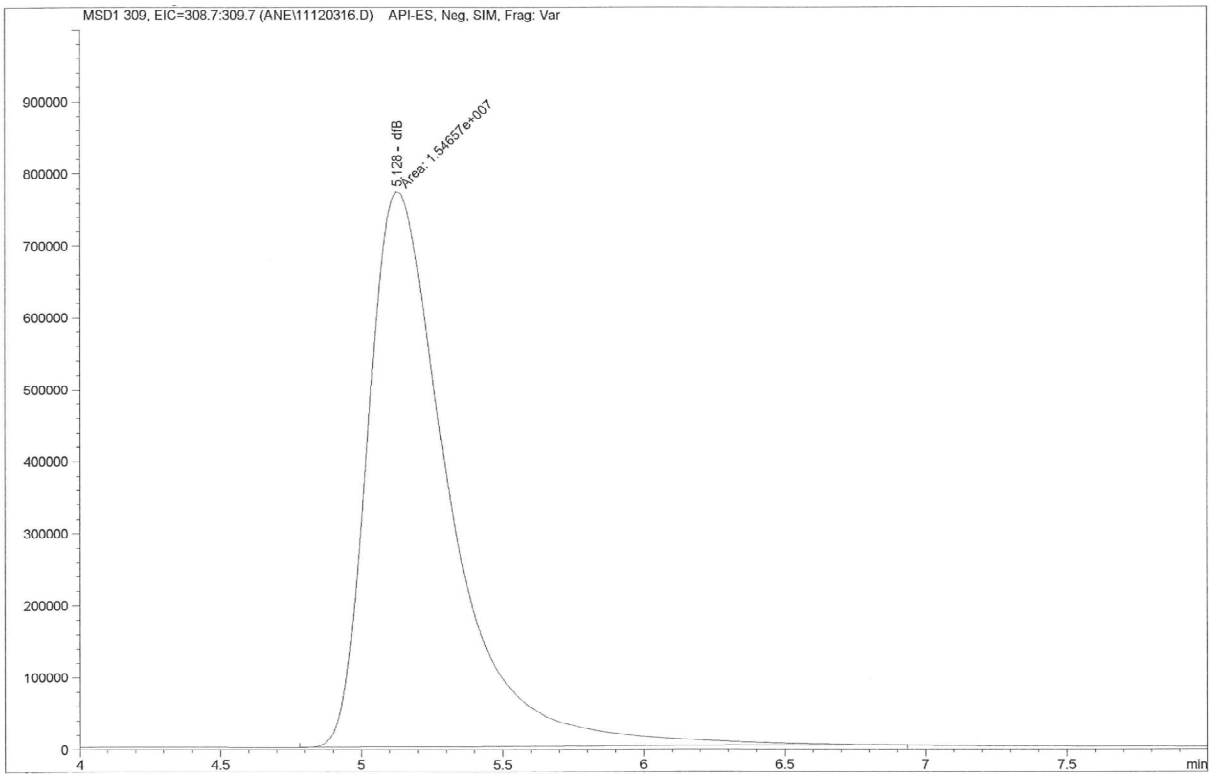


Figure A.22 Colon, sample 150

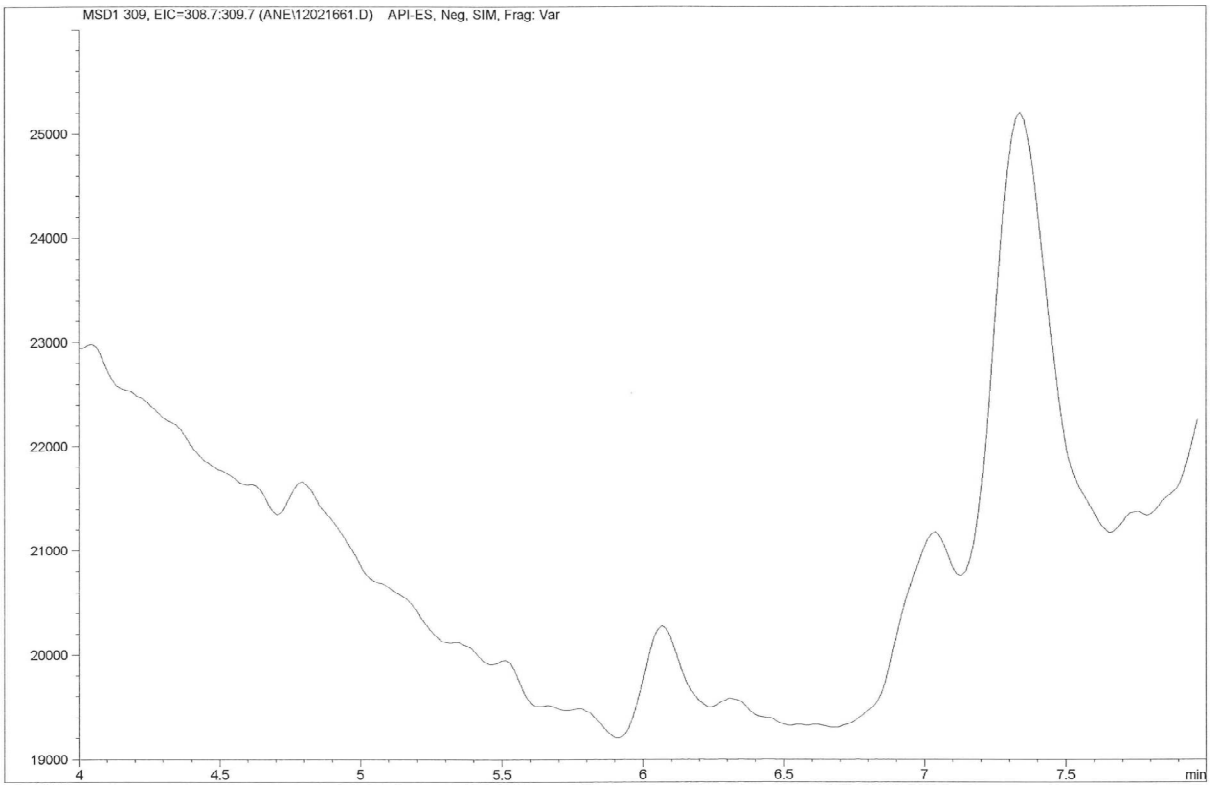


Figure A.23 Bile, samples 110-119 – heptane extraction

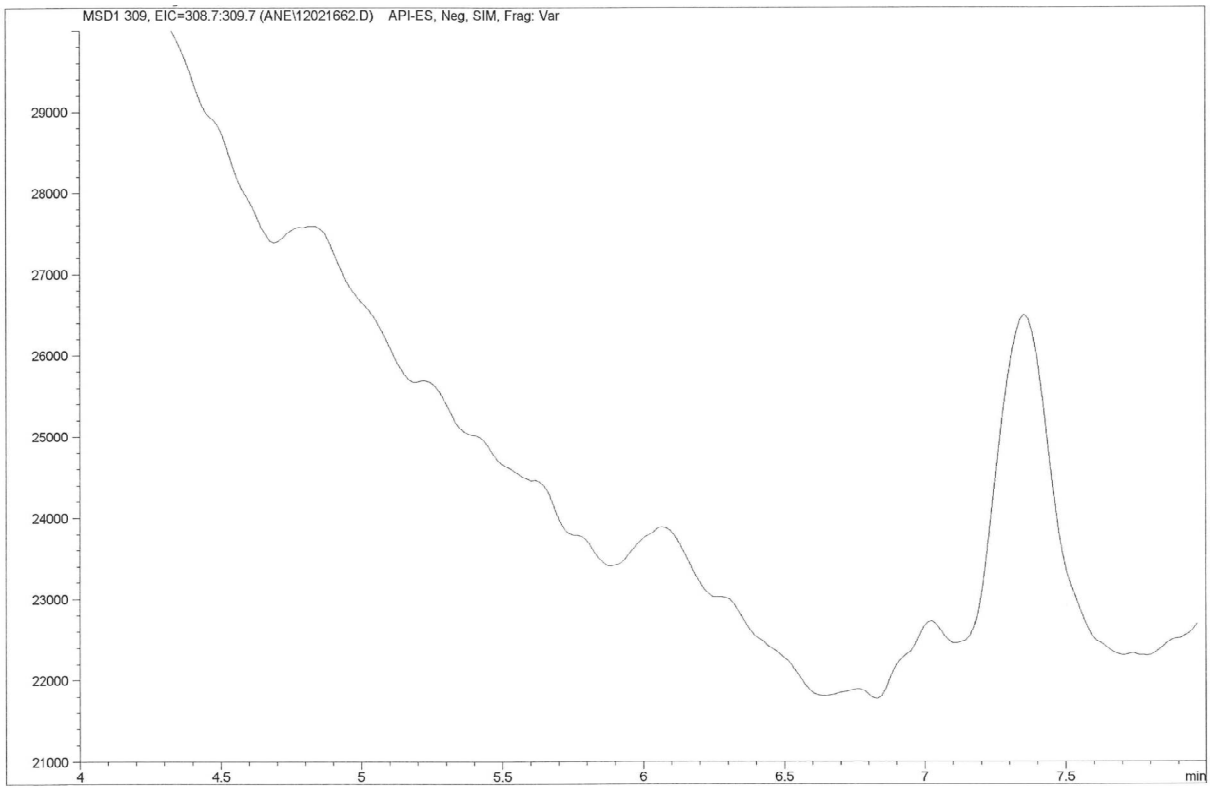


Figure A.24 Bile, samples 130-139 – heptane extraction

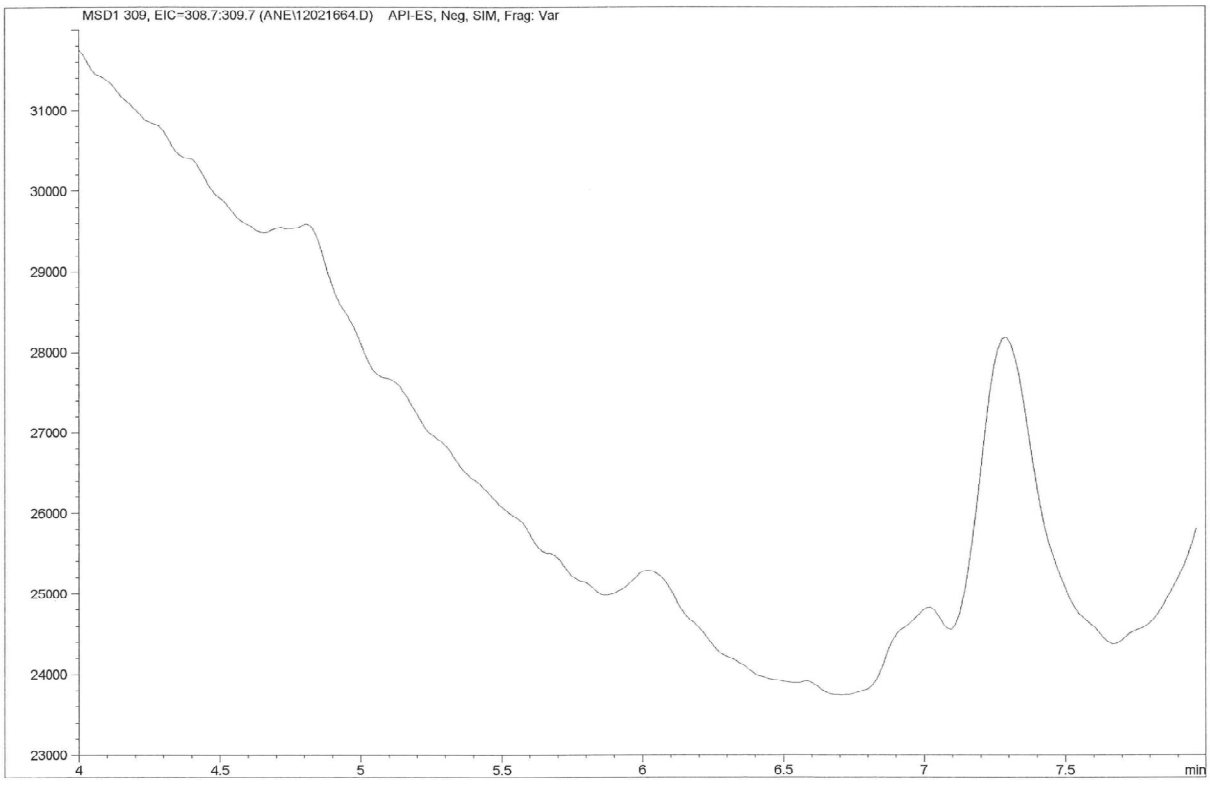


Figure A.25 Bile, samples 140-149 – heptane extraction

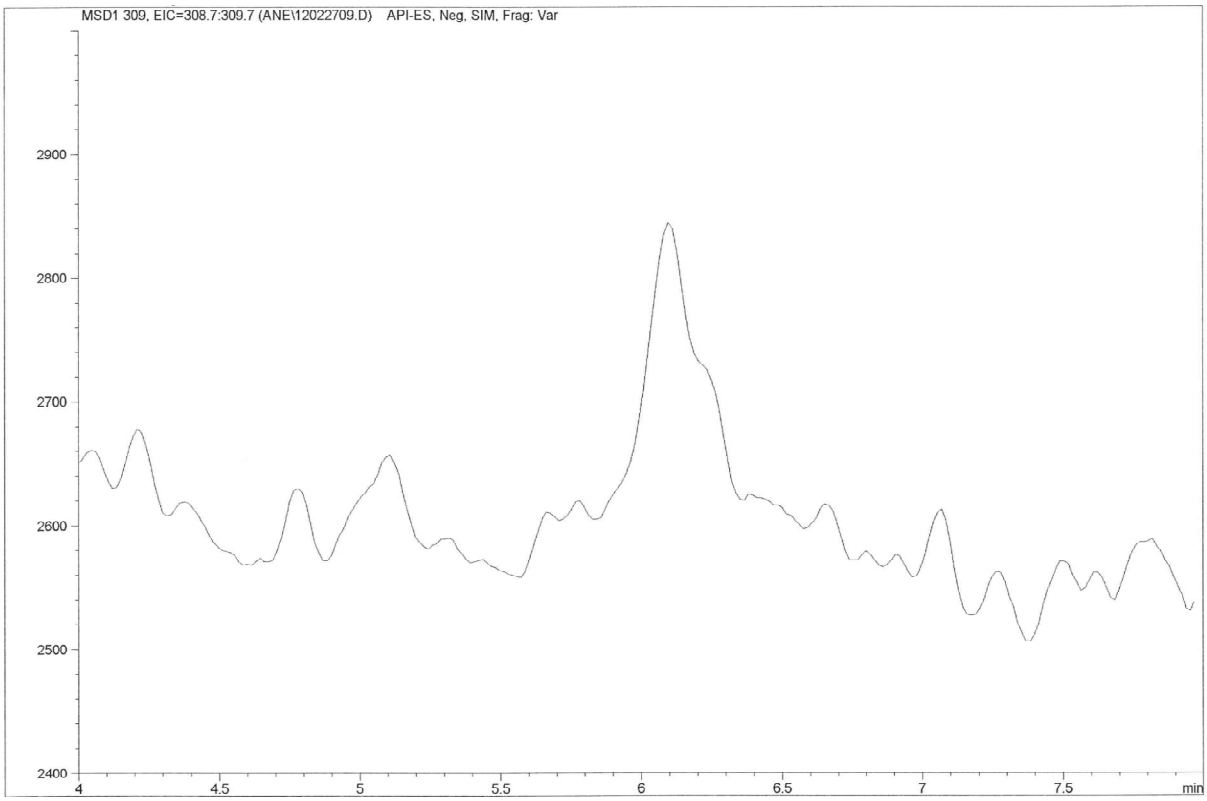


Figure A.26 Bile, samples 110-119 – dichloromethane extraction

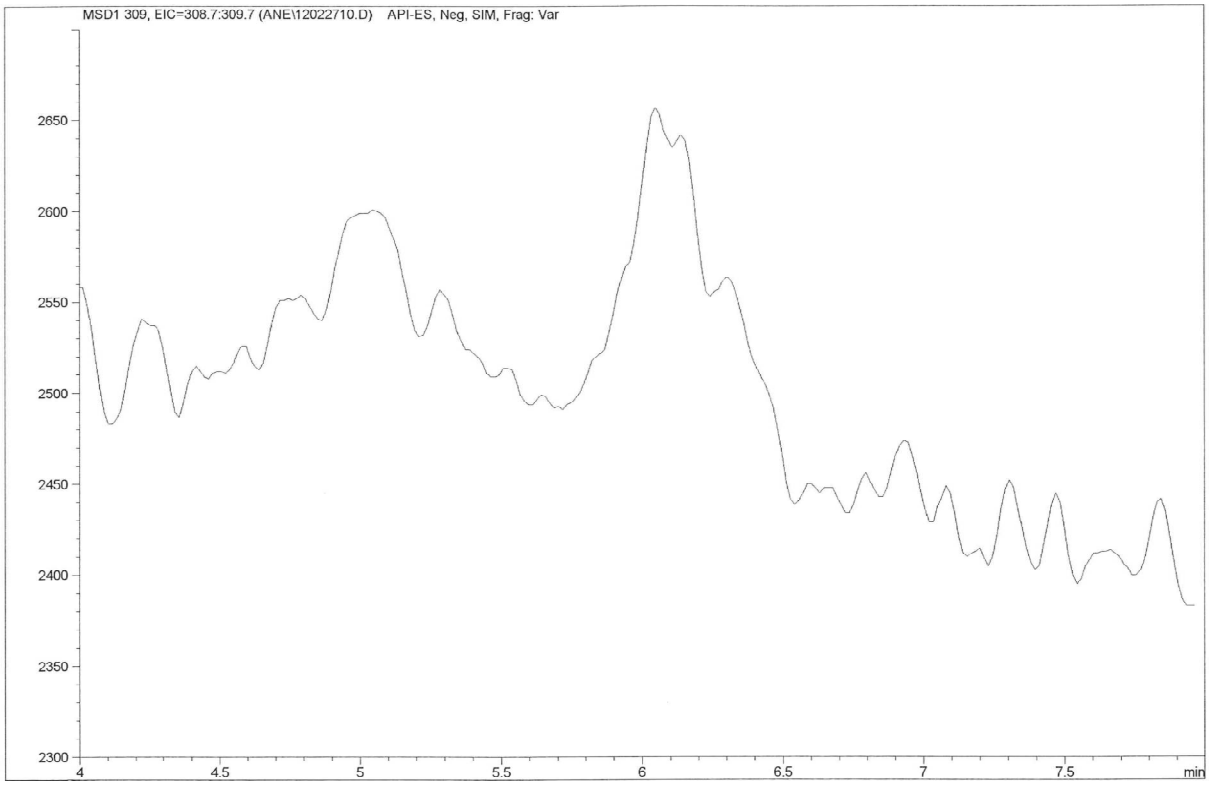


Figure A.27 Bile, samples 130-139 – dichloromethane extraction

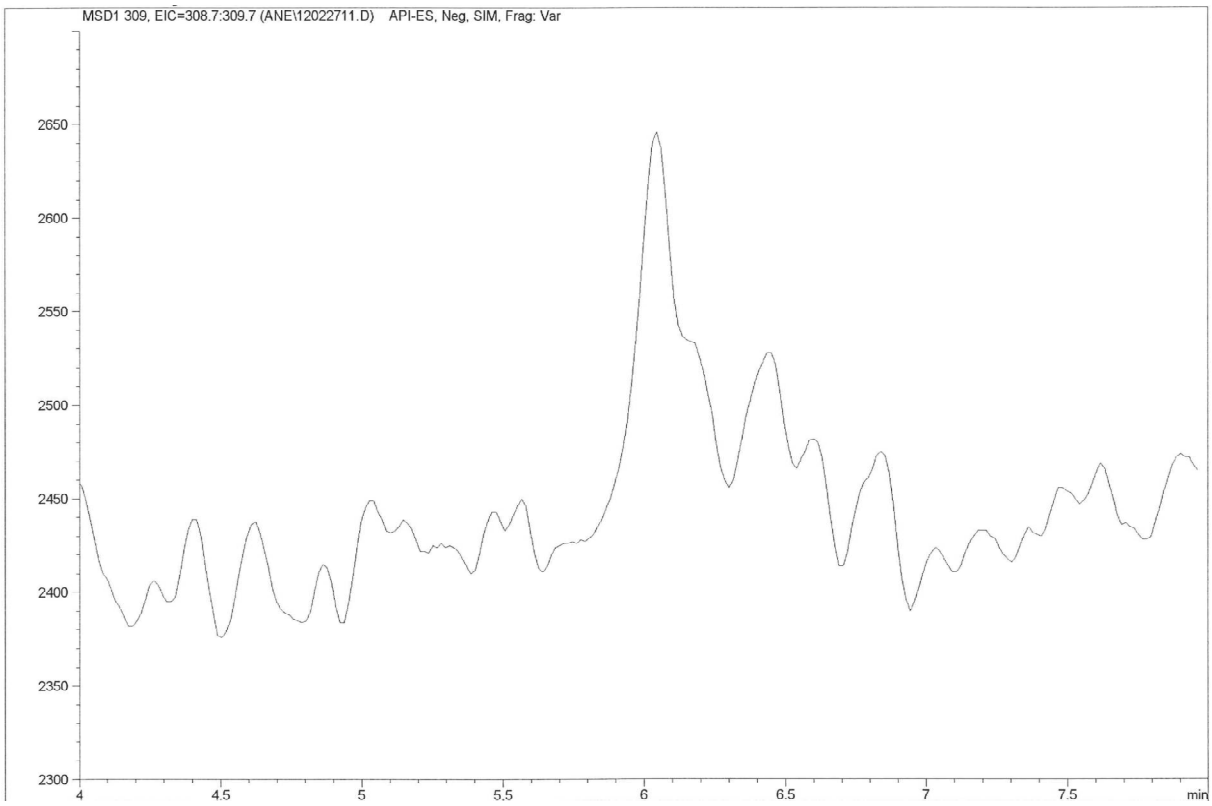


Figure A.28 Bile, samples 140-149 – dichloromethane extraction

Appendix 4 Weight fluctuation of sampled fish

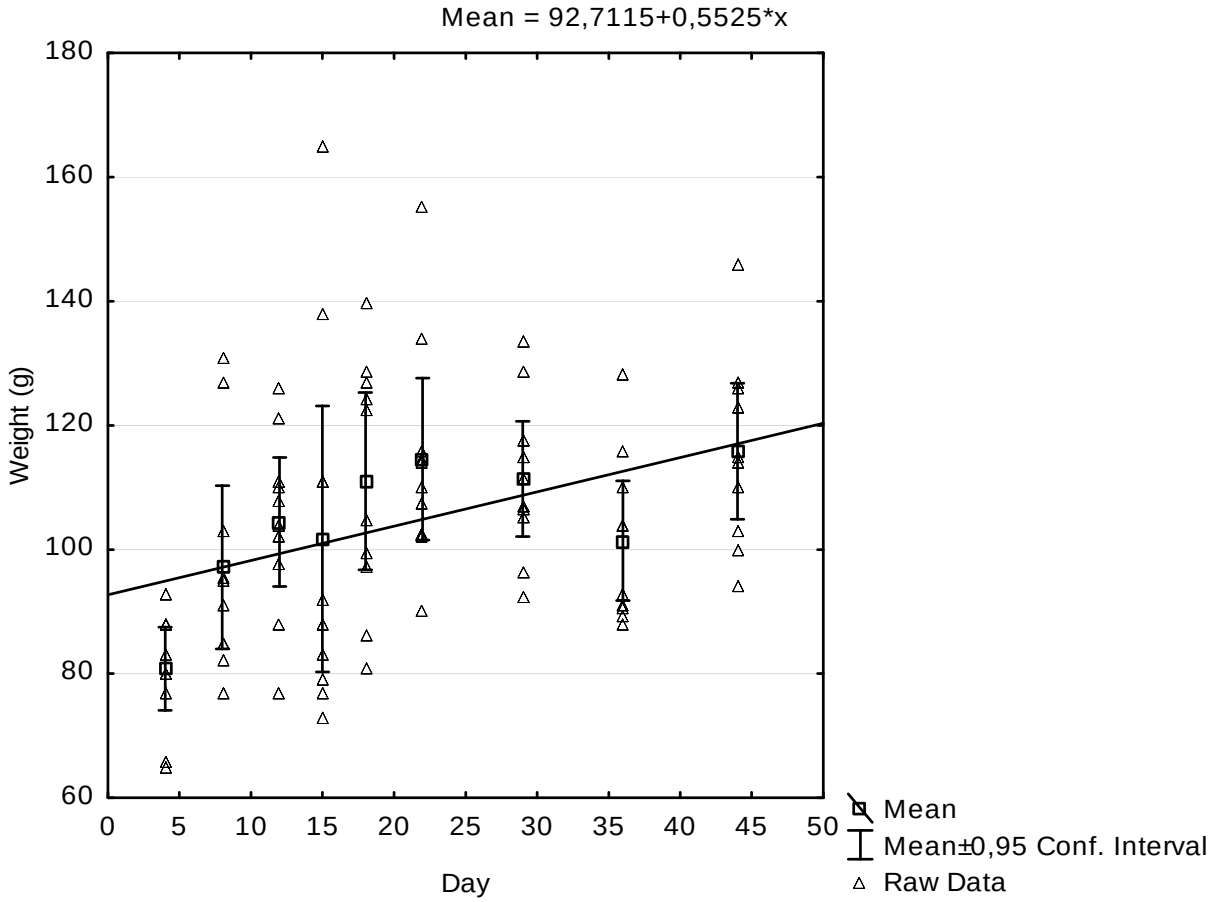


Figure A.29 Weight of fish sampled on each day

Appendix 5 HPLC/MS and HPLC/MS/MS method settings

Method: C:\HPCHEM\1\METHODS\138_DI5.M of 2012/01/23 16:56:24 PM

Method Information

Bestemmelse av diflubenzuron i laks og oerret med teflubenzuron som internstandard.

Method Change History

Operator	Date	Change Information
ttj	2011/08/18 12:57:22 PM	Utvidet opptakstid, 8 min, 5 punkts kurve
aer	2011/09/08 11:00:52 PM	5 punkt standardkurve
eto	2012/01/16 13:26:41 PM	
eto	2012/01/23 16:56:24 PM	

Run Time Checklist

Pre-Run Cmd/Macro:	off
Data Acquisition:	on
Standard Data Analysis:	on
Customized Data Analysis:	off
Save GLP Data:	off
Post-Run Cmd/Macro:	off
Save Method with Data:	off

Method: C:\HPCHEM\1\METHODS\138_DI5.M of 2012/01/23 16:56:24 PM

1100 Quaternary Pump 1

Control

Column Flow	:	0.700 ml/min
Stoptime	:	8.00 min
Posttime	:	Off

Solvents

Solvent A	:	0.0 % (vann)
Solvent B	:	0.0 % (metanol)
Solvent C	:	75.0 % (acetonitril)
Solvent D	:	25.0 % (25 % 10 mM ammuniumhydroksid)

PressureLimits

Minimum Pressure	:	0 bar
Maximum Pressure	:	300 bar

Auxiliary

Maximal Flow Ramp	:	100.00 ml/min^2
Primary Channel	:	Auto
Compressibility	:	100*10^-6/bar
Minimal Stroke	:	Auto

Store Parameters

Store Ratio A	:	Yes
Store Ratio B	:	Yes
Store Ratio C	:	Yes
Store Ratio D	:	Yes
Store Flow	:	Yes
Store Pressure	:	Yes

Timetable is empty

Agilent 1100 Diode Array Detector 1

Signals

Signal	Store	Signal,Bw	Reference,Bw [nm]
A:	No	250 100	360 100
B:	No	254 16	360 100
C:	No	210 8	360 100
D:	No	230 16	360 100
E:	No	280 16	360 100

Spectrum

Store Spectra	:	None

Time

Stoptime	:	As pump
Posttime	:	Off

Required Lamps

UV lamp required	:	No
Vis lamp required	:	No

Autobalance

Prerun balancing	:	Yes
Postrun balancing	:	No

Margin for negative Absorbance: 100 mAU

Peakwidth	:	> 0.1 min
Slit	:	4 nm

Analog Outputs

Zero offset ana. out. 1: 5 %
Zero offset ana. out. 2: 5 %
Attenuation ana. out. 1: 1000 mAU
Attenuation ana. out. 2: 1000 mAU

Timetable is empty

Mass Spectrometer Detector

General Information

Use MSD : Enabled
Ionization Mode : API-ES
Tune File : atunes.tun
StopTime : asPump
Time Filter : Enabled
Data Storage : Condensed
Peakwidth : 0.15 min
Scan Speed Override : Disabled

Signals

[Signal 1]

Polarity : Negative
Fragmentor Ramp : Disabled

Sim Parameters

Time (min)	Group Name	SIM Ion	Frag-mentor	Gain EMV	SIM Resol.	Actual Dwell
0.00		289.00	70	1.0	High	218
		309.00				218
		359.00				218
		378.90				218

Spray Chamber

[MSZones]

Gas Temp : 350 C maximum 350 C
DryingGas : 8.0 l/min maximum 13.0 l/min
Neb Pres : 40 psig maximum 60 psig

VCap (Positive) : 3600 V
VCap (Negative) : 3600 V

[Time Table]

Time Table is empty.

END OF MS ACQUISITION PARAMETERS

FIA Series

FIA Series in this Method : Disabled

Method: C:\HPCHEM\1\METHODS\138_DI5.M of 2012/01/23 16:56:24 PM

Time Setting
Time between Injections : 0.80 min

==========
Agilent 1100 Autosampler 1
==========

Injection
Injection Mode : Standard
Injector volume : 20.0 µl

Auxiliary
Drawspeed : 200 µl/min
Ejectspeed : 200 µl/min
Draw position : 3.0 mm

Time
Stoptime : As Pump
Posttime : Off

==========
Agilent 1100 Column Thermostat 1
==========

Temperature settings
Left temperature : Not controlled
Right temperature : Same as left
Enable analysis : When Temp. is within setpoint +/- 2.0°C
Store left temperature : Yes
Store right temperature: No

Time
Stoptime : As pump
Posttime : Off

Column Switching Valve : Column 1

Timetable is empty

==========
Integration Events
==========

Default Integration Event Table "Event"

Event	Value	Time
Initial Slope Sensitivity	1.000	Initial
Initial Peak Width	0.040	Initial
Initial Area Reject	1.000	Initial
Initial Height Reject	1.700	Initial
Initial Shoulders	OFF	Initial

Detector Default Integration Event Table "Event_DAD"

Event	Value	Time
Initial Slope Sensitivity	5.000	Initial
Initial Peak Width	0.050	Initial
Initial Area Reject	5.000	Initial
Initial Height Reject	1.000	Initial
Initial Shoulders	OFF	Initial

Detector Default Integration Event Table "Event_ADC"

Event	Value	Time
Initial Slope Sensitivity	1.000	Initial
Initial Peak Width	0.040	Initial
Initial Area Reject	1.000	Initial
Initial Height Reject	1.700	Initial
Initial Shoulders	OFF	Initial

Detector Default Integration Event Table "Event_FLD"

Event	Value	Time
Initial Slope Sensitivity	1.000	Initial
Initial Peak Width	0.040	Initial
Initial Area Reject	1.000	Initial
Initial Height Reject	1.700	Initial
Initial Shoulders	OFF	Initial

Detector Default Integration Event Table "Event_MSD"

Event	Value	Time
Initial Slope Sensitivity	10000.000	Initial
Initial Peak Width	0.070	Initial
Initial Area Reject	1000.000	Initial
Initial Height Reject	100.000	Initial
Initial Shoulders	OFF	Initial

Signal Specific Integration Event Table "Event_MSD1SPC"

Event	Value	Time
Initial Slope Sensitivity(Full Scan)	1.000	Initial
Initial Peak Width(Full Scan)	0.250	Initial
Initial Slope Sensitivity(Cond. Scan/SIM)	0.100	Initial
Initial Peak Width(Cond. Scan/SIM)	0.050	Initial
Initial Area Reject	0.000	Initial
Initial Height Reject	5.000	Initial
Initial Shoulders	OFF	Initial

Detector Default Integration Event Table "Event_VWD"

Event	Value	Time
Initial Slope Sensitivity	1.000	Initial
Initial Peak Width	0.040	Initial
Initial Area Reject	1.000	Initial
Initial Height Reject	1.700	Initial
Initial Shoulders	OFF	Initial

Detector Default Integration Event Table "Event_ECD"

Event	Value	Time
Initial Slope Sensitivity	1.000	Initial
Initial Peak Width	0.040	Initial
Initial Area Reject	1.000	Initial
Initial Height Reject	1.700	Initial
Initial Shoulders	OFF	Initial

Detector Default Integration Event Table "Event_MWD"

Event	Value	Time
Initial Slope Sensitivity	1.000	Initial
Initial Peak Width	0.040	Initial
Initial Area Reject	1.000	Initial
Initial Height Reject	1.700	Initial
Initial Shoulders	OFF	Initial

Signal Specific Integration Event Table "Event_MSD1"

Event	Value	Time
Initial Slope Sensitivity	1.000	Initial
Initial Peak Width	0.040	Initial
Initial Area Reject	1.000	Initial
Initial Height Reject	1.700	Initial
Initial Shoulders	OFF	Initial

Signal Specific Integration Event Table "Event_MSD1379"

Event	Value	Time
Initial Slope Sensitivity	1476.631	Initial
Initial Peak Width	0.160	Initial
Initial Area Reject	0.000	Initial
Initial Height Reject	21.324	Initial
Initial Shoulders	OFF	Initial

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Signal Specific Integration Event Table "Event_MSD1359"

Event	Value	Time
Initial Slope Sensitivity	698.365	Initial
Initial Peak Width	0.160	Initial
Initial Area Reject	0.000	Initial
Initial Height Reject	14.514	Initial
Initial Shoulders	OFF	Initial

Signal Specific Integration Event Table "Event_MSD1309"

Event	Value	Time
Initial Slope Sensitivity	2574.493	Initial
Initial Peak Width	0.160	Initial
Initial Area Reject	0.000	Initial
Initial Height Reject	47.779	Initial
Initial Shoulders	OFF	Initial

Signal Specific Integration Event Table "Event_MSD1289"

Event	Value	Time
Initial Slope Sensitivity	1003.440	Initial
Initial Peak Width	0.160	Initial
Initial Area Reject	0.000	Initial
Initial Height Reject	21.801	Initial
Initial Shoulders	OFF	Initial

Signal Specific Integration Event Table "Event_MSD1TIC"

Event	Value	Time
Initial Slope Sensitivity	14000.000	Initial
Initial Peak Width	0.150	Initial
Initial Area Reject	10000.000	Initial
Initial Height Reject	2000.000	Initial
Initial Shoulders	OFF	Initial
Integration OFF		1.000
Integration ON		3.000

Apply Manual Integration Events: No

Advanced Baseline : No

Peak Top Type : parabolic interpolation

Specify Report

Destination: Printer, Screen
Quantitative Results sorted by: Signal
Report Style: dfb
Sample info on each page: No

Signal Options

Include: Axes, Compound Names, Retention Times, Baselines, Tick Marks

Method: C:\HPCHEM\1\METHODS\138_DI5.M of 2012/01/23 16:56:24 PM

Font: Arial, Size: 8

Ranges: Use Ranges

	Min Value	Max Value
Time	1.000	6.000
Response	300.000	1500.000

Multi Chromatograms: Separated, All the same Scale

Calibration Table

Calib. Data Modified : 2012/01/16 13:20:10 PM

Calculate : Internal Standard
Based on : Peak Area

Peak Identification : based on time windows and qualifiers
Rel. Reference Window : 5.000 %
Abs. Reference Window : 4.820 min
Rel. Non-ref. Window : 5.000 %
Abs. Non-ref. Window : 6.210 min
Multiplier : 1.0000
Dilution : 1.0000
Sample Amount : 50.00000
Uncalibrated Peaks : compound name not specified
Partial Calibration : No recalibration if peaks missing

Curve Type : Linear (some peaks differ, see below)
Origin : Ignored (some peaks differ, see below)
Weight : Equal (some peaks differ, see below)

Recalibration Settings:
Average Response : No Update
Average Retention Time: Average all calibrations

Calibration Report Options :
Printout of recalibrations within a sequence:
Calibration Table after Recalibration
Normal Report after Recalibration
If the sequence is done with bracketing:
Results of first cycle (ending previous bracket)

Sample ISTD Information:

ISTD #	ISTD Amount [ng/g]	Name
1	50.00000	ISTD (TFB)

Signal 1: MSD1 379, EIC=378.6:379.6
Signal 2: MSD1 359, EIC=358.7:359.7
Signal 3: MSD1 309, EIC=308.7:309.7
Signal 4: MSD1 289, EIC=288.7:289.7

RetTime [min]	Sig	Lvl	Amount [ng/g]	Area	Amt/Area	Ref	Grp	Name
5.173	3	1	20.00000	3.05016e4	6.55703e-4	1		dfB
		2	35.00000	5.01307e4	6.98174e-4			
		3	50.00000	7.48179e4	6.68289e-4			
		4	60.00000	8.69928e4	6.89712e-4			
		5	75.00000	1.12723e5	6.65347e-4			
6.466	1	1	50.00000	5.71848e4	8.74358e-4	+I1		ISTD (TFB)
		2	50.00000	5.81481e4	8.59874e-4			
		3	50.00000	7.99313e4	6.25537e-4			
		4	50.00000	8.02551e4	6.23013e-4			

d: C:\HPCHEM\1\METHODS\138_DI5.M of 2012/01/23 16:56:24 PM

RetTime [min]	Sig	Lvl	Amount [ng/g]	Area	Amt/Area	Ref	Grp	Name
		5	50.00000	6.33888e4	7.88783e-4			
6.458	2	1	50.00000	2.46337e4	2.02974e-3			
		2	50.00000	2.46294e4	2.03010e-3			
		3	50.00000	3.38345e4	1.47778e-3			
		4	50.00000	3.36171e4	1.48734e-3			
		5	50.00000	2.77176e4	1.80391e-3			

More compound-specific settings:

Compound: dfB
  Time Window : From 4.673 min To 5.873 min
  Curve Type : Linear
  Origin : Forced

  Qualifier on Signal 4: MSD1 289, EIC=288.7:289.7
  Time Window : From 4.620 min To 5.820 min
  Curve Type : Linear
  Origin : Forced

Compound: ISTD (TFB)
  Time Window : From 5.466 min To 7.466 min
  Curve Type : Linear
  Origin : Forced
  Calibration Level Weights:/
    Level 1 : 1
    Level 2 : 1
    Level 3 : 1
    Level 4 : 1
    Level 5 : 1

Peak Sum Table

No Entries in table

Acquisition Method Report

Acquisition Method Info

Method Name 138_DI5.m
Method Path D:\MassHunter\methods\138_DI5.m
Method Description Default Method

Device List
h-ALS-SL
BinPump-SL
Column-SL
MS QQQ

QQQ Mass Spectrometer

Ion Source ESI
Tune File atunes.tune.xml
Stop Mode No Limit/As Pump
Stop Time 1
Time Filter On
Time Filter Width 0.07

Time Segments

Time Seg #	Time	Scan Type	Ion Mode	Div Valve	Delta EMV	Store
1	0	MRM	ESI	To MS	0	☑
2	2.4	MRM	ESI	To MS	0	☑
3	3.4	MRM	ESI	To MS	0	☑

Time Segment 1

Scan Segments

Compound Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Dwell	Frag (V)	CE (V)	Polarity
4-chloranilin	☐	128	Unit	111	Unit	50	110	25	Positive

Source Parameters

Parameter	Value (+)	Value (-)
Gas Temp (°C)	300	300
Gas Flow (l/min)	6	6
Nebulizer (psi)	15	15
Capillary (V)	3500	3500

Time Segment 2

Scan Segments

Compound Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Dwell	Frag (V)	CE (V)	Polarity
Diflubenzuron	☐	311	Unit	158	Unit	50	110	30	Positive
Diflubenzuron	☐	311	Unit	141	Unit	50	110	20	Positive
4-chloranilin	☐	128	Unit	111	Unit	50	110	25	Positive

Source Parameters

Parameter	Value (+)	Value (-)
Gas Temp (°C)	300	300
Gas Flow (l/min)	6	6
Nebulizer (psi)	15	15
Capillary (V)	3500	3500

Time Segment 3

Scan Segments

Acquisition Method Report

Compound Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Dwell	Frag (V)	CE (V)	Polarity
Teflubenzuron	☑	379	Unit	359	Unit	50	100	0	Negative
Teflubenzuron	☑	379	Unit	339	Unit	50	100	10	Negative
Teflubenzuron	☑	379	Unit	316.1	Unit	50	100	14	Negative
Teflubenzuron	☑	379	Unit	294.9	Unit	50	100	14	Negative
Teflubenzuron	☑	379	Unit	195.9	Unit	50	100	18	Negative

Source Parameters

Parameter	Value (+)	Value (-)
Gas Temp (°C)	300	300
Gas Flow (l/min)	6	6
Nebulizer (psi)	15	15
Capillary (V)	3500	3500

Chromatograms

Chrom Type	Label	Offset	Y-Range
TIC	TIC	0	10000000

Instrument Curves

Actual

#N/A

Wellplate Sampler

Name h-ALS-SL **Model** G1367C
Ordinal # 1 **Options** THM

Stop Time (min) As Pump **Post Time (min)** Off

Injection Type Needle Wash
Overlap Time Disable Overlapped Injection
Draw Position Detection 0
Eject Speed 200
Automatic Delay Volume Reduction No
Wash Vessel N/A
Wash Time 1
Ready Temp. Range

Injection Volume 5
Draw Position -2
Draw Speed 200
Flush Out Factor 5
Equilibration Time 0
Wash Location FlushPort
Wash Cycles N/A
Temp.

Contact 1 0
Contact 2 0
Contact 3 0
Contact 4 0

Injector Program
Signals Selected
Contacts Time Table

Binary Pump

Name BinPump-SL **Model** G1312B
Ordinal # 1 **Options** SSV

Stop Time (min) 6 **Post Time (min)** Off

Flow (ml/min) 0.25 **Pressure Min (bar)** 0

Pressure Max (bar) 600 **Max Flow Gradient (ml/min)** 100

Solvent A **Solvent B** 1% maursyre
Solvent Ratio A 75 **Solvent Ratio B** 25

Solvent Type A1
Solvent Type A2
Solvent Type B1
Solvent Type B2

Compress. A (*10-6/bar) 100
Compress. B (*10-6/bar) 115

Stroke A Auto
Stroke B Auto

Stroke Syncronization

Contact 1 0
Contact 2 0
Contact 3 0
Contact 4 0

Pump Time Table

Time	Flow	Pressure	Solv Ratio B
0	0.25	600	25
6	0.25	600	25

Signals Selected
Contacts Time Table

Thermostated Column Compartment

Name Column-SL **Model** G1316B
Ordinal # 1 **Options** CSV

Stop Time (min) As Pump **Post Time (min)** Off

Left Temp. Not Controlled
Left Ready When Temp Within Set Point +/- 0.8
Valve Position 1

Right Temp. Same as left
Right Ready When Temp Within Set Point +/- 0.8

Contact 1 0
Contact 2 0
Contact 3 0
Contact 4 0

Acquisition Method Report

Temperature Time Table
Signals Selected

Description
Temperature of left heat exchanger
Contacts Time Table